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STUDIES ON THE FUNDULUS CHORION [1]

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The developing embryos of *Fundulus heteroclitus* have served as material for permeability studies since their use by Loeb ('08, '12, '14–'16, '22) in his classic studies on permeability and salt antagonisms. Two membranes of differing nature are involved in the penetration of substances into the embryo: the external *chorion* and the *ectoderm* of the embryo itself. The chorion is a tough, transparent, resilient protein membrane about 12 μ thick. The outer 7–8 μ appear sieve-like with 10–20,000 pores per square millimeter, the pores being about 1.5 μ in diameter. The inner 3–4 μ of the chorion is composed of densely packed fibrils in a concentric meshwork. On extrusion from the oviduct there are many hair-like fibrils forming a loose external felting on the chorion. A micropyle is readily seen. A number of workers prior to 1900 have reported observations on the structure of the egg membranes of fish (Ryder, 1885; Mark, 1890; Balfour and Parker, 1881, 1882; Schultze, 1856; Calberla, 1878; Lereboullet, 1854; and Hoffman, 1881).

Between the chorion and the embryo is the subchorionic space, which accounts for 25–35% of the internal volume of the chorionic shell, the remainder being taken up by the embryo and yolk. With the closure of the blastopore, the embryo and yolk are covered by a continuous layer of ectoderm which remains unbroken until the mouth and gills open shortly before hatching.

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The original experiments of Loeb did not define the respective roles of the chorion and the ectoderm in the penetration of substances into the embryo. This present report is limited to a consideration of some of the permeability properties of the chorion, firstly, some osmotic properties of the chorion, and secondly, the migration of hydrogen ions across the chorion as modified by salts.

Osmotic behavior

The osmotic properties were observed under several sets of conditions. When eggs are placed in concentrated solutions of sucrose, a depression develops in the chorion which increases until the chorion rests against the body of the embryo. It became apparent that this developing depression might serve as an index of the chorion's permeability to water. Measurement of this process furnishes an index of the exosmosis of water.

In addition, when the eggs with depressed chorions are placed in dilute solutions, or water, the spherical shape is restored. Measurement of this process furnishes an index to the endosmosis of water.

The exosmosis of water also can be determined by carefully introducing a fine capillary micropipette into the subchorionic space, and attaching this to a calibrated manometric device containing water. These eggs were then immersed in various media. It was observed that the chorion did not collapse, but rather the level of water in the manometric device fell at a steady rate. It is possible to compute the volume of water which has passed out through the chorion.

When whole eggs are placed in a molal solution of sucrose in water, within a few seconds the depression has a diameter half that of the chorion. This was taken as the end point because of the ease of observation and the computation of the volume change. These same indented eggs were then placed in distilled water and the time required for their restoration to a sphere was determined. This latter process

terminated with a visible "snap" of the chorion. This terminal "snap" was not as striking in those embryos in which the hatching mechanisms had been operative (Armstrong, '36).

Davson and Danielli ('43) define the permeability of a membrane to a substance as the *net* mol transfer per liter across a membrane of one square micron in one second per each gram-mol difference in gradient. With this a basis for calculation, the exosmosis when the depression is formed averages 1.1×10^{-15} mols/sec./μ^2.

The volume of water removed from within the chorion by the production of the depression, at the end point noted above, is given by the formula:

$$V = \pi/3\ h(3r_a + h^2)$$

where r_a is the radius of the depression and equals half the radius of the sphere. The height of the chord, h, is given by the formula:

$$h = r - r_a \sqrt{3.}$$

With the mean radius of the chorion 0.9 mm the volume of water exchanged in the formation of the depression is 0.0324 mm^3. One gram-mol of water occupies about 18 cm^3. The formula for the surface area of a sphere is $4\pi r^2$. The basic formula to convert these data into a transfer rate is:

$$\text{mols/area} \times \text{time} \times \text{gradient}$$

The average time was 20 seconds for the formation of the depression to the end point. Table 1 gives the mean values of time involved, the gradients, and the calculated mean mol transfer rates.

There is a difference in the gradient across the chorion in the depression and restoration responses of the chorion, a factor of some 11 times. The restoration, however, occurs at a rate twice that expected if the rate were dependent on the gradient alone. This apparent paradox is resolved in part by a consideration of the "snap" of complete restoration and the slow response of choria during early phases of hatching. These choria are resilient membranes, and one may poke at

them with needles and they will rebound away into the fluid with remarkable swiftness. Their behavior in this manner is analogous to plastic *ping-pong* balls.

There seems to be a point in the restoration where the tension of the chorion becomes sufficient to restore the spheroid outline without benefit of an osmotic gradient. With many pores in the chorion, there is little resistance to this rapid movement of the *sieve,* which the chorion thus resembles. This higher rate of endosmosis may be explained by a concentration of the salts in the subchorionic space during the exosmosis of water in the formation of the depression,

TABLE 1

Mean water transfer rates in mols

SITUATION	WATER GRADIENT	MEAN TIME COURSE	TRANSFER RATES
	mols	*sec.*	$\times 10^{-15}$
Depression in sucrose	7.8	20 ± 3	1.2
Restoration in water	0.7	100 ± 15	2.5
Manometric device	7.8	arbitrary	2.8

relatively reducing the ratio of the gradients, and changing the transfer rate to a value closer to 1.2×10^{-15}, that of the exosmosis of water. Whether both mechanisms play a role, or in what proportion these are of import, was not further studied.

Sucrose passes the chorion. Cryoscopic data suggest that the subchorionic concentration is the same as the external medium after about an hour. If the eggs are left in the sucrose media the depression will be lost with restoration of the spheroid shape of the chorion, in a time course of 50–75 minutes. After 6 to 20 hours further exposure, the yolk sac becomes crenated, then the heart ceases to beat, and the embryo dies. Sometimes the eggs float when they are first placed in the sucrose media of higher density. Shortly they too sink, and about this same time the spheroid shape of the chorion has been restored and analysis of the subchorionic

fluid suggests that here again, the concentration of sucrose in the subchorionic space equals that of the external medium.

Other solutions were tested for similar effects. Glycerol was made up to provide the same water gradient as molal sucrose and its effect was the same, except that restoration was more rapid. Sucrose in molal proportions in sea water caused a similar sequence of events, but restoration was slower. Exposure times up to 17 hours in this medium failed to kill any of the embryos. For the same osmotic effect of molal sucrose in sea water a solution of two molal sucrose in distilled water was used. Here restoration required about 4 hours. After 22 hours the yolk sac became crenated and the chorion was again depressed. This occurred without killing any embryos.

It makes no difference relative to the behavior of choria in sucrose media whether the embryo died in early development or not. There are no differences between freshly stripped eggs, whether fertilized or not. In these terms the embryo has no regulatory function over the osmotic behavior of the chorion. In this connection a paper by Kao and Chambers ('54) is significant. They observed changes in the internal hydrostatic pressure at the time of lysis of the yolk platelets during the first stages of activation. With strong sucrose solutions about the chorion no subchorionic space develops. This presumably is due to a balance of forces. The lysis of platelets normally is accompanied by a shrinkage of the yolk sac, so probably the water, and possibly other substances come from within the sac. A number of other investigators have considered the problem of water balance at the *Fundulus* chorion. Svetlov ('29) considered the osmotic pressure changes of the egg to be due largely to changes in the content of the subchorionic space. Brown ('03, '07) found he could not set up a gradient of electric potential and considered this due to a membrane whose permeability to ions was so great as to preclude electrical gradients.

The chorion of *Salmo fario* is similar. Bogucki ('30) showed that these eggs develop in hypertonic media. Gray

('20, '32) also sensed an analogy for these two species from work on the trout egg. By an ingenious method he was able to show a rapid change in the subchorionic space with modification of the surrounding medium. The shell of *Salmo* is 90 μ thick and heavy. When the solution was light to the yolk, the yolk would sink and rest on the bottom of the shell, while if the yolk were light to the solution, the yolk would float up and rest against the inside of the apex of the shell.

Facilitation of hydrogen ion passage across the chorion by various chloride salts

It was shown by Armstrong ('27, '28) that the pH of the subchorionic fluid approximates that of the surrounding medium whether this latter is sea water, distilled water, or a chemical solution. This change in pH is a gradual one. This may be followed by injecting colorimetric pH indicator dyes into the subchorionic fluid during the change at frequent intervals.

The comparative rates of acid penetration had previously been inferred by observing the necrosis of the ectoderm and the cessation of the heart beat that follows the entry of hydrogen ions into the egg. Loeb noted a delay in these toxic effects by the addition of various salts to the acid. He postulated that this action was at the chorion. Armstrong, using dechorionated embryos of the same age as controls, suggested that the major site of the antagonism was at the ectoderm. The problem of the separation of the membranes to determine the site of action is largely a technical one. The mature eggs range from 1.7 to 2.1 mm in diameter and techniques for the removal of the chorion were not devised (Nicholas, '27; Armstrong, '27) until after Loeb had done his work on salt antagonisms. He used newly hatched fry as controls. These latter present to the various media and the natural environment some membrane surfaces other than the ectoderm; viz., the gill clefts and oral cavity. In order to

understand the ectodermal antagonism, the role of the chorion needed clarification.

Five to 9-day embryos were used. At these stages the subchorionic space occupies about 25–35% of the total volume of the egg and ample fluid is available for pH determinations. For comparative purposes Loeb's basic concentrations of salts and acids were used. The acetic acid was a constant M/500 solution, and the chlorides of potassium, calcium, and sodium were used in various strengths. The pH changes in acid alone and in combination with various salts were measured.

In preliminary experiments it was found that 30 minutes were required for the pH of eggs fresh from sea water to become that of distilled water. These were then placed in a dilute borate buffer of pH 8.4 which was that of the sea water used. The process of re-adjustment took about 60 minutes. With these data as the basis, the technique developed was to leach the eggs for 90 minutes in several changes of distilled water, and then to place them in the dilute buffer for another 90 minutes to restore the native pH of the chorion and subchorionic space. This had no discernible effect on the embryos.

The pH of the M/500 acetic acid was computed to be 3.73 and measured as 3.75 with bromphenol blue as the indicator dye. The end point used was 3.9, largely for the reason that the yellow color of the yolk seemed to interfere with readings beyond that point. Table 2 gives the various times observed for the various media to reach this end point.

For each individual medium, a plot of the pH changes yields a sigmoid curve which was similar for all media, differing principally in slope. The salt effect, possibly, is in an abundant supply of ions to fill positions of charge on the protein matrix of the chorion which otherwise might require hydrogen ions, thus allowing a freer passage of the latter. In this respect sea water itself is effective. There is a small pH carrier effect of the salt media, which is approximately that of distilled water itself. The rate of diffusion of hydrogen

ion, using the Davson formula, ranges from 2.7 to 5.5 $\times$ 10^{-17} mols/sec./μ^2.

These data show a qualitative similarity between the *Fundulus* chorion and Loeb's gelatin coated collodion membrane. The significance of his work, then, is in the basic antagonism of salts and acids at the ectoderm, for these data show there is no delay of hydrogen ion passage in the presence of salts. There is a delay in the sense that the ions reach the ectoderm later than they would if the chorion were not at all present. In this regard the chorion tends to protect the em-

TABLE 2

The observed time for the subchorionic fluid to reach the pH end point of 3.9

SUBCHORIONIC FLUID	EXTERNAL MEDIUM	MINUTES
Sea water	acid	55
Buffer	acid	75
Buffer	acid, M/16 NaCl	70
Buffer	acid, M/32 NaCl	70
Buffer	acid, M/64 NaCl	70
Buffer	acid, M/128 NaCl	75
Buffer	acid, M/16 KCl	45
Buffer	acid, M/32 KCl	55
Buffer	acid, M/64 KCl	70
Buffer	acid, M/1024 $CaCl_2$	45
Buffer	acid, M/2048 $CaCl_2$	60
Buffer	acid, M/4096 $CaCl_2$	60

bryo from changes in the environment, although the chemical changes occur so rapidly that the chorion probably serves significantly only as a mechanical protection for the embryo.

In 1928 Sumwalt pointed out that Armstrong's data did not show that the ectoderm was the *exclusive* site of antagonism. The present study establishes that fact. The ectoderm is the sole site of the salt-acid antagonism, and probably the site of all regulatory processes between egg and environment. Sodium-22 radiotracer was found to equilibrate across the chorion in the absence of a sodium gradient in about one hour, a rate sufficiently rapid to have significance on salt balance at the ectoderm (Shanklin, unpublished). Gray's

work with the fresh water *Salmo* suggests that if any difference exists between fresh and salt water forms in respect to this behavior, then this is more subtle than these methods of analysis can determine.

SUMMARY AND CONCLUSIONS

A description of the structure of the chorion is presented. Water readily passed the chorion in both directions. Sucrose, glycerol and sodium ion pass the chorion, and to an equilibrium concentration equal to that of the external environment. The age of the egg after closure of the blastopore and whether the egg is alive or not have no effect on the passage of water, except that after a weakening of the chorion by the hatching enzyme the responses differ somewhat. Prolonged exposure to strong osmotic solutions leads eventually to crenation and death of the embryo. Hydrogen ion passes the chorion, and after a brief time the pH of the subchorionic space is the same as the external medium. Salt solutions accelerated this passage of hydrogen ion and in general the acceleration increases with the strength of the salt solution. The site of the salt-acid antagonism observable in the intact egg is therefore at the ectoderm.

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LITERATURE CITED

Armstrong, P. B. 1927 Determinations of the pH of developing Fundulus eggs. Proc. Soc. Exp. Biol. Med., *25:* 146–147.

———— 1928 The antagonism between acetic acid and the chlorides of sodium, potassium, and calcium as manifested in developing Fundulus embryos. J. Gen. Physiol., *11:* 515–523.

———— 1936 Mechanism of hatching in Fundulus heteroclitus. Biol. Bull., *71:* 407.

Balfour, F. M., and W. N. Parker 1881 On the structure and development of Lepidosteous. Proc. Roy. Soc. Lond., *33:* 112–119.

———— 1882 On the structure and development of Lepidosteous. Phil. Trans. Roy. Soc. Lond., Part II, 359–442.

Bogucki, M. 1930 Recherches sur la Perméabilité des membranes et sur la pressium osmotique des oeufs des salmonides. Protoplasma, *9:* 345.

Brown, O. H. 1903 The immunity of Fundulus eggs and embryos to electrical stimulation. Am. J. Physiol., *9:* 111–115.

———— 1907 The permeability of the egg of Fundulus heteroclitus. Ibid., *14:* 354–358.

Calberla, E. 1878 Der Befruchtungsvorgang beim Ei von Petromyzon Planeri. Zeitschr. f. wiss. Zool., *30:* 437–486.

Davson, H.. and J. F. Danielli 1943 The Permeability of Natural Membranes. Macmillan Co., New York.

Gray, J. 1920 The relation of the animal cell to electrolytes. I. A physiological study of the egg of the trout. J. Physiol., *53:* 308–319.

———— 1932 The osmotic properties of the eggs of the trout. J. Exp. Biol., *9:* 277–299.

Hoffmann, C. K. 1881 Zur Ontogenie der Knochenfische. Verhandl. a. koninkl. Alsad. v. Witenschappen, 21, Amsterdam.

Kao, C. Y., and R. Chambers 1954 Internal hydrostatic pressure of the Fundulus egg. I. The activated egg. J. Exp. Biol., *31:* 139–149.

Lereboullet, A. 1854 Résumé d'un Travail d'Embryologie comparée sur le Développement der Brochet, de la Perche et de l'Ecrevisse. Ann. Sci. Nat. ser. 4 Zool. Tan I, 237–289.

Loeb, J. 1908 A new proof of the permeability of cells for salts or ions, a preliminary communication. Univ. Calif. Pub. Physiol., (1905–1910) *3:* 81–86.

———— 1912 Antagonistic action of electrolytes and permeability of the cell membrane. Science, N.S., *36:* 637–639.

———— 1914 Is the antagonistic action of salts due to oppositely charged ions? J. Biol. Chem., *19:* 431–433.

———— 1915 On the role of electrolytes in the diffusion of acid into the egg of Fundulus. Ibid., *23:* 139–144.

———— 1916 The mechanism of the diffusion of electrolytes through the membranes of living cells. I. The necessity of a general salt effect upon the membrane as a prerequisite for this diffusion. Ibid., *27:* 339–352.

———— 1916a The mechanism of the diffusion of electrolytes through the membranes of living cells. II. The diffusion of KCl out of the egg of Fundulus, and the relative efficiency of different ions for the salt effect. Ibid., *27:* 353–362.

———— 1916b The mechanism of the diffusion of electrolytes through the membranes of living cells. III. The analogy of the diffusion for acids and potassium salts. Ibid., *27:* 363–375.

LOEB, J. 1917 The mechanism of the diffusion of electrolytes through the membranes of living cells. V. The additive effect of salt and base and the antagonistic effect of salt and acid. Ibid., *32:* 147–158.

———— 1922a Sodium chloride and selective diffusion in living organisms. J. Gen. Physiol., *5:* 231–254.

———— 1922b The influence of salts on the rate of diffusion of acid through collodion membranes. Ibid., *5:* 255–264.

LOEB, J., AND H. WASTENEYS 1915 Note on the apparent change of the osmotic pressure of cell contents with the osmotic pressure of the surrounding solution. J. Biol. Chem., *23:* 157–162.

MARK, E. L. 1890 Studies on Lepidosteus, Bull. Mus. Comp. Zool., *19:* 1.

NICHOLAS, J. H. 1927 The application of experimental methods to the study of developing Fundulus embryos. Proc. Nat. Acad. Sci., *13:* 695–698.

RYDER, J. A. 1885 On the development of viviparous osseous fishes and of the Atlantic salmon. Proc. U. S. Nat. Mus., 128–162.

SCHULTZE, M. S. 1856 Die Entwickelungsgeschichte von Petromyzon Planeri. Eine von der hollandischen Societät der Wissenchaften zu Haarlem, i, J.

SUMWALT, M. 1933 Ion effects upon ion permeability of the Fundulus chorion. Biol. Bull., *64:* 114–123.

SVETLOV, P. 1929 Entwicklungsphysiologische Beobachtungen an Forelleneiern. Arch. f. Entwicklungsmechanik, *114:* 771.

EVIDENCE OF ERYTHROCYTE INCOMPATIBILITY IN PIGEONS OF AN INBRED STRAIN USING RADIOCHROMIUM-TAGGED CELLS [1]

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ONE FIGURE

Although it is well known that blood from one human being may produce severe reactions when transfused into another, such reactions receive little attention among the lower animals. In practice blood from individual dogs, rats, guinea pigs and rabbits is given intravenously to others of the same species with little expectation of transfusion reactions. Similarly such procedures have been used in our laboratory on pigeons and ducks with no outwardly apparent reactions. Data have been collected, however, which show that erythrocytes of one individual pigeon may have a very short survival when transfused into another individual of the same strain, and the data demonstrating this incompatiblity are the subject of this report.

MATERIALS AND METHODS

Pigeons of the White Carneau strain, 6 weeks of age when received, were used throughout the study. The birds were maintained on Purina Pigeon Chow and water, ad libitum.

One ml of blood was removed from the wing vein by venipuncture, and was drawn into a syringe containing 1 ml ACD[2] solution. The diluted blood was gently delivered into a 50-ml

[1] This investigation was supported in part by a research grant C-1500 from the National Cancer Institute, National Institutes of Health, Public Health Service; and American Cancer Society Grant No. IN-1.

[2] Abbott Laboratories, special A-C-D Solution, containing: dextrose, 132 mg; sodium citrate, 250 mg; citric acid, 80 mg; water, 10 ml.

beaker to which was added 0.5 ml of a diluted radiochromium-51 solution. Dilutions of the stock solution, as sodium chromate, were made such that 0.5 ml contained 20–24.5 μc of radioactivity and 0.81–0.96 μg of chemical chromium. The mixture was incubated at room temperature for 20 minutes with occasional, gentle agitation, and 10 mg of *l*-ascorbic acid were added to reduce the hexavalent chromium to the trivalent form to prevent further tagging of the erythrocytes (Read, '53). The mixture was then injected intravenously into designated birds. Twenty-four hours later a 0.1 ml sample was drawn from the leg vein of each bird, and at appropriate intervals thereafter. Each sample was placed in a small plastic vial containing 4 ml of physiological saline. The Cr^{51} activity of the samples was determined, and the survival times of the erythrocytes derived as reported previously (Marvin and Lucy, '57).

RESULTS AND DISCUSSION

In table 1 the results are summarized, with the birds receiving isologous[3] blood grouped according to the maximal survival times. Of the 12 birds receiving blood from other individuals, three or 25% had normal, 5 or 42% had moderately reduced, and 4 or 33% had severely shortened maximal survival times, as compared with the average value for the 16 tests using autologous transfusions.

In 10 cases birds were transfused isologously, then after the tagged cells had not been detected for 4 weeks, each bird was transfused with its own blood tagged with chromium, thus serving as its own control. This procedure was carried out in order to eliminate the possibility that certain birds may have an excessive, intrinsic, hemolytic process. It should be emphasized that none of the birds appeared to be deleteriously affected by the injection of blood from other individuals. Also of importance is the fact that none of the birds had

[3] The term autologous refers to injections of blood into the same individual from which it was obtained; isologous, into another individual of the same inbred strain.

TABLE 1

Survival times of autologously and isologously tranfused erythrocytes

RECIPIENT-AUTOLOGOUS				DONOR-AUTOLOGOUS				ISOLOGOUS		
BIRD NO.	Survival times			BIRD NO.	Survival times			Survival times		
	Max.[1]	Mx.[1]	50%[1]		Max.	Mx.	50%	Max	Mx.	50%
9	—	—	—	36A	47	11.0	8.0	46	16.0	10.0
36B	46	11.5	9.3	117	46	14.5	10.0	47	8.2	6.6
90	46	14.0	12.5	36A	47	11.0	8.0	45	12.8	8.0
46	41	14.5	8.5	31	46	12.3	12.2	35	13.0	7.0
55	41	22.5	11.5	36A	47	11.0	8.0	32	15.2	7.8
62	46	15.0	9.5	44	53	20.0	11.0	30	11.4	5.8
94	37	7.2	9.8	30	40	6.5	5.5	22	5.8	3.0
98	43	13.4	12.2	117	46	14.5	10.0	28	9.7	5.0
41	—	—	—	127	47	13.3	10.9	16	3.8	2.5
68	41	14.2	9.4	30	40	6.5	5.5	14	5.4	2.9
79	46	22.5	12.0	44	53	20.0	11.0	16	9.2	4.9
85	46	23.2	11.5	30	40	6.5	5.5	15	8.0	4.3

[1] Max. refers to maximal survival time, Mx. to mean survival time, and 50% refers to time for disappearance of 50% of tagged cells, all values in days.

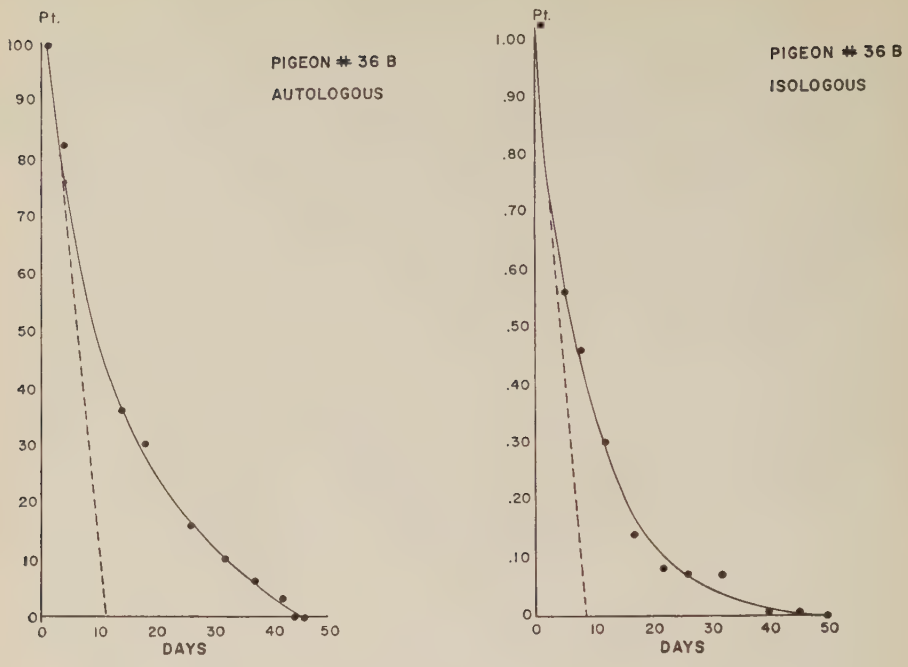

Figure 1A

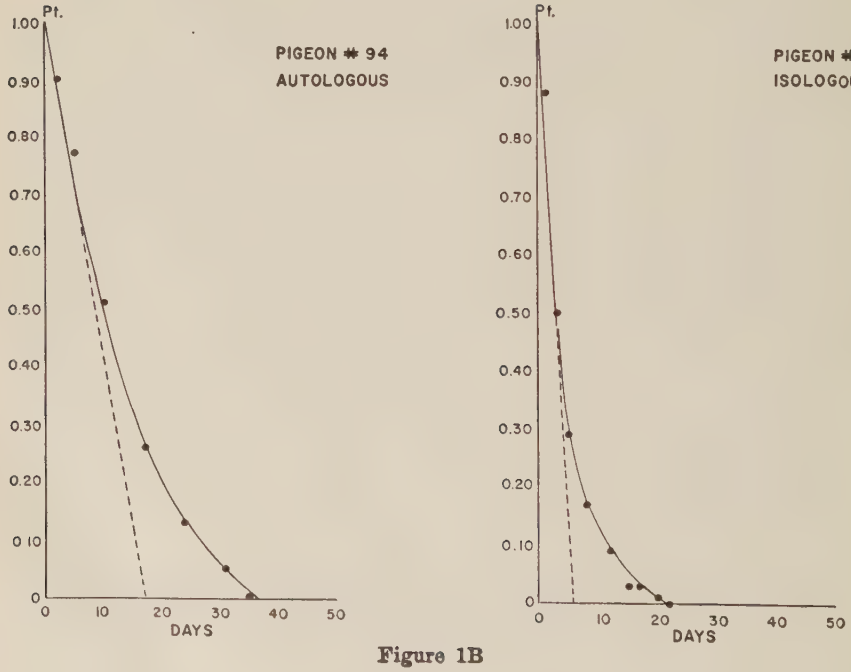

Figure 1B

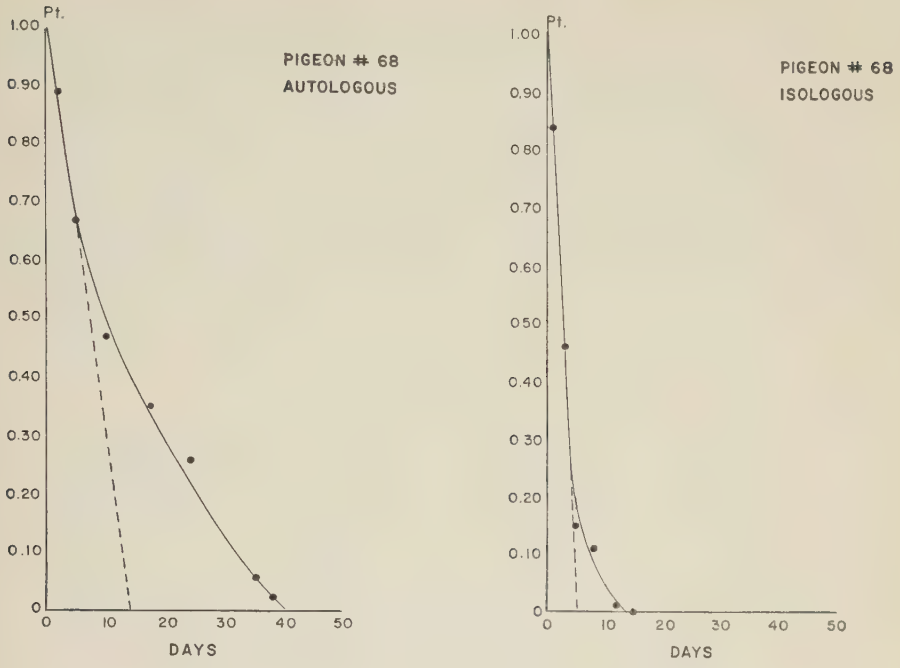

Figure 1C

Fig 1 Erythrocyte survival curves of three pigeons showing on the left the autologous survival, and on the right the isologous survival of cells. Figure 1A represents an instance of normal survival isologously, figure 1B a case of moderately shortened, and figure 1C an instance of severely shortened survival.

been treated in any fashion before receiving the blood of another bird.

Although subject to considerable variation within groups, the mean survival and 50% survival times were correlated with the maximal survival times. The greater variability of the first two statistics, as compared to maximal survival times, has been reported previously (Marvin and Lucy, '57).

The presence of immunologically demonstrable antigens in the erythrocytes of pigeons and pigeon-dove hybrids has been reported by Irwin and his group (reviewed by Irwin, '55). Normal bovine serum agglutinates the erythrocytes of chickens, and it has been found that chickens of several families fall into three classes based on their capacity to react (Briles,

Briles and Irwin, '52). By procedures similar to those used by Irwin ('55), McGibbon ('44) has demonstrated antigens in the erythrocytes of ducks, species specific for the Mallard and Muscovy strains. Thus there seems to be ample evidence that erythrocyte antigens exist in several species of birds. The data reported herein strongly suggest that transfusion incompatibilities exist between individuals of a single strain of pigeons.

TABLE 2

Comparison of survival times of donor blood isologously transfused into several hosts

DONOR BIRD	RECIPIENT BIRD	MAX. RBC SURVIVAL	
		Group	Days
36A	9	Normal	46
	90	Normal	45
	55	Moderate	32
117	36B	Normal	47
	98	Moderate	28
44	62	Moderate	30
	79	Short	16
30	94	Moderate	22
	68	Short	14
	85	Short	15

In table 2 of some of the data is grouped to show that the survival of cells from one pigeon will differ, depending upon the pigeon into which it is injected. It is noteworthy that in no case did the injected blood have widely different survival times in recipient birds. That is, the donor blood had either normal and moderate, or moderate and short survival times; not normal and short. This fact may be significant immunologically, and subject to resolution by those techniques.

As reported previously by Rodman, Ebaugh and Fox ('57) and confirmed by Marvin and Lucy ('57), the number of tagged cells has a curvilinear relationship to time in days. In figure 1 are shown representative curves of red cell survival of both autologous and isologous transfusions of the three

levels of survival; namely: normal, moderately shortened, and severely shortened. The extended initial slopes of the curves and their intercepts with the time axis are shown, which is the method of Dornhorst ('51) for determining the mean survival time. From an inspection of the curves, it can be seen that there is a quantitative difference between the autologous and isologous curves, but not an obvious qualitative difference. This would suggest that the incompatibility is really an acceleration of existing process, rather than an introduction of a drastically new process.

SUMMARY

Erythrocytes of pigeons were tagged with radioactive chromium and injected intravenously into the individual bird (autologously) from which they were obtained. Under this circumstance, the cells were found to have an average mean survival of 12.3 days, a maximal survival of 46.0 days, and 50% had disappeared in 8.8 days. When the tagged cells were injected into a bird of the same strain other than the donor (isologously), the survival was shortened significantly in 75% of the tests. These findings are taken as evidence of transfusion incompatibility within a single strain of pigeons.

LITERATURE CITED

Briles, W. E., R. W. Briles and M. R. Irwin 1952 Differences in specificity of the antigenic products of a series of alleles in the chicken. Genetics, *37:* 359–368.

Dornhorst, A. C. 1951 The interpretation of red cell survival curves. Blood, *6:* 1284–1292.

Irwin, M. R. 1955 Biological Specificity and Growth, III. Immunogenetics. Princeton University Press, pp. 55–71.

Marvin, H. N., and D. D. Lucy 1957 The survival of radiochromium-tagged erythrocytes in pigeons, ducks and rabbits. Acta Haem., *18:* 239–245.

McGibbon, W. H. 1944 Cellular antigens in species and species hybrids in ducks. Genetics, *29:* 407–419.

———— 1945 Further division of contrasting antigens in species hybrids in ducks. Ibid., *30:* 252–265.

Read, R. C. 1953 A simplified procedure for the measurement of red cell volume and turnover with Cr^{51}. Surg. For., *4:* 169–172.

Rodnan, G. P., F. G. Ebaugh, Jr. and M. R. S. Fox 1957 The life span of the red blood cell and the red blood cell volume in the chicken, pigeon and duck as estimated by the use of $Na_2Cr^{51}O_4$. Blood, *12:* 355–366.

TREHALASE IN THE THORACIC MUSCLES OF THE WOODROACH, LEUCOPHAEA MADERAE

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FOUR FIGURES

Recently Wyatt and Kalf ('56) discovered that the hemolymph of a number of different insects contains relatively large amounts of the disaccharide trehalose (α-D-glucopyranosyl-α-D-glucoypyranoside). This sugar, until then, was known to occur only in lower plants, especially in fungi. Previously Frerejacque ('41) and Duspiva ('54) had reported that the digestive tract of certain insects is able to split trehalose into glucose. In a few preliminary experiments Frerejacque ('41) also showed the presence of a specific trehalase in insects.

Considering the apparent significance of trehalose in the hemolymph of insects, based on the large amounts found by Wyatt and Kalf ('56), it was of interest to study the enzyme which acts specifically upon this sugar.

MATERIAL AND METHODS

Thoracic muscle of the woodroach, *Leucophaea maderae,* which contains a very active trehalase was used for most of the experiments. The tissue was homogenized with a Potter-Elvehjem homogenizer. Then the homogenate was frozen because this treatment proved to increase the trehalase activity (table 2) probably by facilitating the access of the substrate to the enzyme. The reaction mixture was incubated at 37°C

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and shaken continuously. At the end of the incubation time $Ba(OH)_2$ and $ZnSO_4$ were added. This proved to be sufficient to stop the reaction immediately. After centrifugation of the precipitate the formed glucose was determined in the supernatant filtrate by the method of Somogyi as modified by Nelson ('44).

Reaction products

It had been suggested by Frerejacque ('41), and Wyatt and Kalf ('56) that the hydrolysis of trehalose possibly may be coupled with a phosphorylation step similar to the hydrolysis of sucrose observed in some bacteria (Doudoroff et al., '48):

Sucrose + inorganic phosphate ⇌
glucose-1-phosphate + fructose

If this is true for insect trehalase, it would mean that this enzyme belongs to the group of the disaccharide phosphorylases. In order to find out whether other reaction products besides glucose had been formed in the hydrolysis of trehalose the reaction mixtures were analyzed by means of paper chromatography for sugars (Trevelyan et al., '50) and sugar phosphates (Mortimer, '52). The results were negative as it was not possible to detect any other compound except glucose arising from the hydrolysis of trehalose. It seems probable, therefore, that insect trehalase acts like an ordinary hydrolase.

Specificity and inhibition

The specificity of insect trehalase is demonstrated in table 1. The enzyme hydrolyzed sucrose and maltose only to a very small extent. Even after long incubation (24 hrs.) most of the sucrose remained unchanged while maltose appeared to be hydrolyzed to a somewhat greater extent. Both sugars had an inhibitory effect on the hydrolysis of trehalose. Thus, when sucrose and maltose were present in the same concentration as trehalose they inhibited about 40% of the activity obtained with trehalose alone. Lactose was not attacked by muscle homogenate of the woodroach and neither did it inhibit the hydrolysis of trehalose.

TABLE 1

Hydrolysis of trehalose by homogenate of woodroach muscle and the influence of some other sugars on it

Trehalose	+	+	+	+	−	−	−	−
Sucrose	−	+	−	−	+	−	−	−
Maltose	−	−	+	−	−	+	−	−
Lactose	−	−	−	+	−	−	+	−
Glucose ($\times 10^{-6}$ M)	3.89	2.39	2.66	4.16	0.39	1.000	0.17	0.11

0.2 ml 10% homogenate; 2.14×10^{-5} M substrates; reaction medium: 0.033 M phosphate buffer, pH 6.0; incubation time: 60 minutes.

Properties

The pH optimum of the woodroach trehalase was found to be at 6.0 (fig. 1). The enzyme appears to be rather stable for the speed of its action remained constant for several hours (fig. 2). The amount of glucose formed from trehalose is linearly related to the concentration of the enzyme (fig. 3).

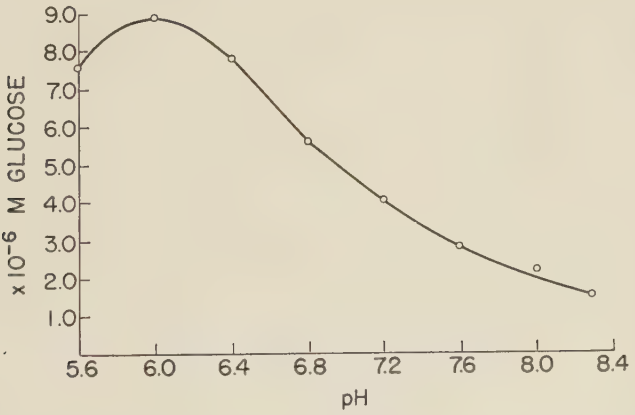

Fig. 1 Influence of pH on the hydrolysis of trehalose by woodroach muscle homogenate; 2.14×10^{-5} M trehalose; reaction medium: 0.033 M phosphate buffer, pH 6.0; incubation time: 90 minutes.

The extraction of the enzyme from the tissue was attempted. The muscles were alternately homogenized, frozen and extracted with a tenfold volume of isotonic KCl solution. This

procedure was repeated 5 times. The extracts showed high trehalase activity which decreased only slightly from one extract to the next. The residue after 5 extractions still contained very high activity, showing that in spite of the efficient

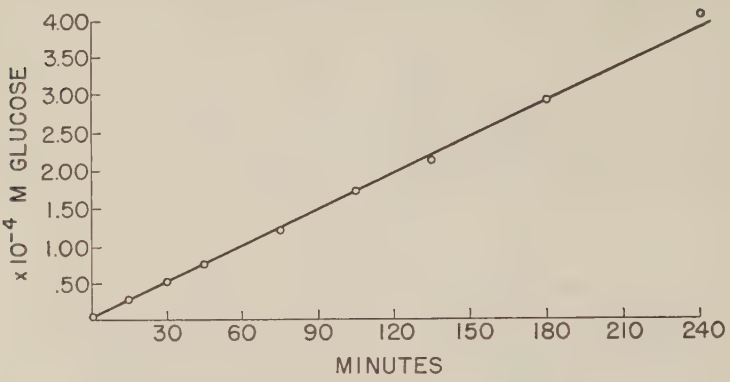

Fig. 2 Trehalose hydrolysis by homogenate of woodroach thoracic muscle as a function of time; 2.0 ml 10% homogenate; 5.40×10^{-4} M trehalose; reaction medium: 0.033 M phosphate buffer, pH 6.0.

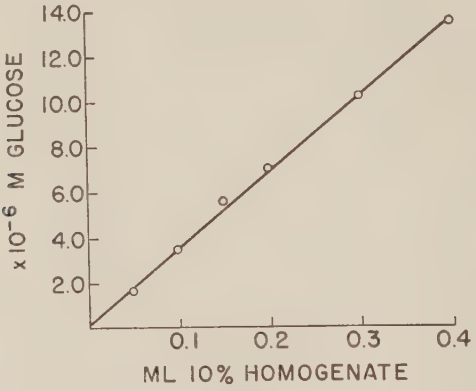

Fig. 3 Trehalose hydrolysis by homogenate of woodroach thoracic muscle as a function of enzyme concentration; 5.35×10^{-6} M trehalose; reaction medium: 0.033 M phosphate, pH 6.0; incubation time: 60 minutes.

extraction procedure used insect trehalase remained to a large extent in the particulate fraction of the cell (table 2).

In this connection it may be pointed out that the influence of phosphate on the hydrolysis of trehalose by the residue of

woodroach muscle was also studied. It was supposed that probably a great part of the phosphate content of the tissue had been removed by the 5 extractions. One would expect, therefore, that the concentration of the remaining phosphate would become the limiting factor in the hydrolysis of trehalose, if this reaction was coupled with a phosphorylation. Also a great difference in the formation of glucose should be found according to whether phosphate was added or omitted. It was demonstrated in parallel experiments with phosphate buffer and citrate buffer, respectively, that exactly the same yield of

TABLE 2

Hydrolysis of trehalose by whole homogenates, different extracts, and residues from woodroach thoracic muscle

	M glucose/ml preparation
	$\times 10^{-5}$
Whole homogenate, unfrozen	1.94
(10%) frozen	2.82
Extract No. 1	0.91
Extract No. 2	0.89
Extract No. 3	0.66
Extract No. 4	0.37
Extract No. 5	0.27
Residue (10%)	3.80

0.2–0.6 ml preparation, 2.14×10^{-5} M trehalose; reaction medium: 0.033 phosphate buffer, pH 6.0; reaction time: 90 minutes.

glucose was obtained by the action of the residue, thus confirming the results reported above by means of another method.

Localization

In order to find out something more definite about the localization of the trehalase within the cell, the mitochondria of the muscles were isolated (Sacktor, '53) and tested for their trehalase activity. A preparation of mitochondria which proved to be essentially microscopically pure contained very high activity. Thus mitochondria have to be considered as one seat of the trehalase activity in insect muscle. Whether it may

also be found in other elements of the cell appears still uncertain since several attempts to obtain highly pure preparations of these fractions of the cell have not been successful.

Trehalose synthesis

It was of interest to determine whether under certain conditions the α-glucosidase which hydrolyzes trehalose is also able to act in the reverse direction, in other words, to synthesize trehalose from glucose. Muscle homogenate was incubated

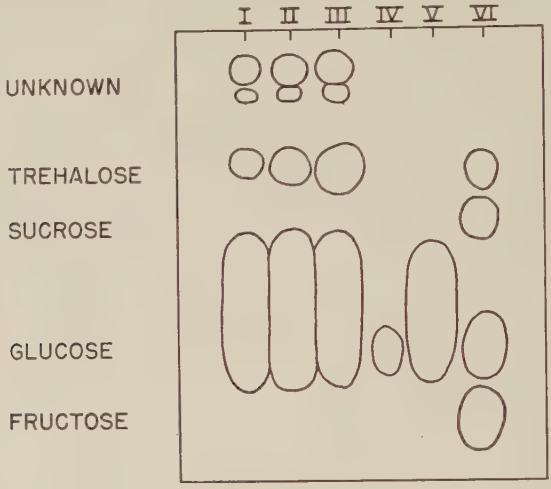

Fig. 4 Chromatogram of the reaction mixtures obtained after incubation of woodroach thoracic muscle homogenates with 20% glucose in 0.132 M phosphate buffer, pH 6.0. I, after 24 hrs.; II, after 48 hrs.; III, after 96 hrs.; IV, control, substrate omitted; V, control, homogenate omitted; VI, pure sugar solutions for comparison. Solvent: butanol-acetic acid-water (4:1:5). Spots developed with $AgNO_3$ and alcoholic NaOH.

with 20% glucose solution and 0.132 M phosphate buffer, pH 6.0. The reaction mixture which had been kept in an atmosphere saturated with toluene fumes was analyzed by means of paper chromatography (Trevelyan et al., '50) after 24, 48 and 96 hours. The chromatograms showed spots which had exactly the same R_f as the pure trehalose of the control mixture (fig. 4). The size of these spots increased with the

incubation time. This finding seems to prove that woodroach muscle has not only the ability to hydrolyze trehalose, but also to synthesize it from glucose. In addition to the spots which probably were identical with trehalose, two others were detected whose R_f was much lower than that of trehalose. They have not yet been identified. Possibly they are indicating the formation of sugars which consists of more than two glucose units. Such oligosaccharides have been found to be formed by the action of other glucosidases (Duspiva, '54; Crook and Stone, '57). In experiments where muscle homogenate was incubated with maltose similar spots with a very low R_f were found.

Distribution

Trehalase activity was found also in other tissues of the woodroach. The data obtained from the fat-body were rather low. No activity was detected in hemolymph. Extremely

TABLE 3

Hydrolysis of trehalose and sucrose by the different parts of the woodroach gut and their contents

	× 10^{-6} M GLUCOSE		
	Fore-gut	Mid-gut	Hind-gut
Epithelium			
Substrate: Trehalose	13.07	3.50	0.06
Sucrose	5.16	6.12	0
Control	0	0	0.11
Contents			
Substrate: Trehalose	12.80	13.1	3.80
Sucrose	12.90	13.1	1.50
Control	0.11	0.11	0.28

2.14×10^{-5} M trehalose and sucrose; reaction medium: 0.033 M phosphate buffer, pH 6.0; incubation time: 60 minutes.

high values were measured in the epithelium of the fore-gut from which the content had been removed. They were twice as high as those of invertase activity (expressed in terms of sucrose hydrolysis; table 3). The content of the fore-gut

which was suspended and extracted showed equally high activities of both enzymes. This indicates that trehalase is not only found within the cells, but is also secreted into the lumen of the digestive tract. In the epithelium of the mid-gut the trehalase activity was significantly decreased while the level of activity remained unchanged in its content. No activity was found in the hindgut epithelium while the content still showed some remaining activity.

Experiments on insects belonging to such different groups as Diptera (*Phormia regina*), Hymenoptera (honey-bee) and Lepidoptera *(Prodenia eridania, Rothschildia forbesi)* gave results very similar to those obtained with the woodroach. This seems to be of special interest since, for instance, bees and moths as adults very likely do not have any opportunity to take up trehalose with their food.

SUMMARY

The homogenate and extract of thoracic muscle of the woodroach, *Leucophaea maderae,* catalyze the hydrolysis of trehalose. This hydrolysis is a result of the action of a specific trehalase. This enzyme shows optimum activity at pH 6, is localized to a large extent in the mitochondria, can catalyze the synthesis of trehalose from glucose, and is also found in the epithelium and the content of the fore-gut and mid-gut. Insects belonging to such different groups as Diptera, Hymenoptera and Lepidoptera gave results similar to those obtained with the woodroach.

ACKNOWLEDGMENT

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LITERATURE CITED

Crook, E. M., and B. A. Stone 1957 The enzymic hydrolysis of β-glucosides. Biochem. J., *65:* 1–12.

Doudoroff, M., N. Kaplan and S. Z. Hassid 1948 Phosphorolysis and synthesis of sucrose with a bacterial preparation. J. Biol. Chem., *148:* 67–75.

DUSPIVA, F. 1954 Enzymatische Prozesse bei der Honigtaubildung der Aphiden. Verh. Deutsch. Zool. Ges., Tübingen, pp. 440–447.

FREREJACQUE, M. 1941 Trehalose et trehalase. C. R. Acad. Sci., Paris, *213:* 88–90.

MORTIMER, D. C. 1952 Paper chromatographic separation of some biologically important phosphate esters. Can. J. Chem., *30:* 653–660.

NELSON, M. 1944 A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., *153:* 375–380.

SACKTOR, B. 1953 Investigations on the mitochondria of the house fly, *Musca domestica* L., I. Adenosintriphosphatases. J. Gen. Physiol., *36:* 371–387.

WYATT, G. R., AND G. P. KALF 1956 Trehalose in insects. Fed. Proc., *15:* 388.

———— 1957 The chemistry of insect hemolymph: II. Trehalose and other carbohydrates. J. Gen. Physiol., *40:* 833–847.

ATROPINE EFFECTS ON THE EMBRYONIC RAT HEART[1]

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TWO FIGURES

There is diversity of opinion as to the nature of action of atropine on cardiac muscle. It is stated in some texts that atropine has slight and insignificant direct effects, in others that small doses stimulate but that large doses depress. Nearly all authors agree that, in blocking acetylcholine (ACh) effects, the drug acts not upon the nerve endings but directly on the muscle fiber. These statements can evidently be tested by experiments on the embryonic (pre-neural) heart.

In the present investigation, it was observed that atropine had no chronotropic or inotropic effects on the embryonic rat heart, that low concentrations were effective in blocking high ACh concentrations, but that these blocking effects were transitory and could no longer be demonstrated within 45 minutes.

MATERIALS AND METHODS[2]

The materials and methods were in general those of previous investigations (Hall, '51, '54, '55, '57). The 11½-day rat embryos were removed singly from the female to Krebs-Ringer solution at body temperature, where the hearts were dissected out under the binocular dissecting microscope.

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[2] It is a pleasure to acknowledge the assistance of Mr. David T. Lewis, Mr. William P. Hale and Mr. Edward M. Bowles, Jr., student research scholars, in the course of this investigation.

The hearts were then transferred to fresh solution for initial control determinations, which were continued until the contraction rate had stabilized. This initial control solution (approximately 360 ml, like the succeeding solutions) was contained in and completely filled a finger bowl, which was covered with a square of plate glass to prevent loss of CO_2 and consequent rise of pH. The preparation was thus essentially a tissue culture with so large an amount of medium that changes in its composition due to the activity of the tissue were precluded.

During observation in the finger bowl and transfer from one solution to the next, the heart rested at the bottom of a container consisting of a shortened glass vial, whose very small capacity (0.15–0.35 ml) ensured that only minimal quantities of fluid and of dissolved drugs were transferred.

The contraction rate was determined by timing 50 beats with a stop-watch each minute, or more frequently when rapid changes in rate were occurring. Fisher's t values were calculated to determine whether differences between average contraction rates were statistically significant. Amplitude of contraction was frequently estimated, and recorded on an arbitrary scale.

A total of 30 control hearts were maintained in Krebs-Ringer without drugs for 80 minutes; the average rate during the first 5-minute period was not significantly different from that during the last 5-minute period.

"Concurrent ACh control" hearts (section IV) were studied after every third or fourth experimental heart to test the effectiveness of the ACh solution. When these "control" hearts occasionally failed to react in typical fashion, the immediately preceding experimental cases were discarded, and a fresh stock ACh solution was prepared. At the end of a day's experimentation, the female rat was subjected to the test for chromodacryorrhea (Tashiro, Smith, Badger and Kezur, '40) as a biological assay of the stock ACh solution.

The stock solution of ACh was kept at about pH 5 to prevent the spontaneous hydrolysis that occurs at higher pH levels.

Experimental ACh solutions were made up immediately before use from stock solutions. Both ACh bromide and chloride were used.

The ACh concentration used experimentally was purposely high (40 mg/l), so that the blocking action of atropine sulphate (0.15 and 0.6 mg/l) could be tested against pronounced ACh effects.

The aneural character of these embryonic hearts has been established by silver impregnations (Hall, '51); recent observations (Hogg, '57) have confirmed and extended these findings.

EXPERIMENTAL

Four types of experiment with atropine were performed, as follows:

I. With the concurrent use of ACh

Thirty hearts were studied individually. After excision of the hearts from the embryos, initial control determinations were made for 5 minutes; the average of these was 172/minute.

The hearts were then transferred to a Krebs-Ringer solution containing 0.15 mg/l of atropine sulphate and 40 mg/l of ACh for 5 minutes, where 16 of the hearts were arrested, usually within the first minute, for 17 seconds on the average. Most of the remaining 14 hearts decelerated briefly, usually within the first minute, and when the slowest rates of these 14 were selected, their average was found to be 115/minute.

Graph A of figure 1 is a composite graph which takes into account all of the rate determinations (usually 6–8) made during the experimental period; this graph shows that recovery of the contraction rate occurred while the hearts were still in the experimental solution, beginning with the second minute in this solution.

The hearts were finally placed in the terminal control solution for 5 minutes, where the average rate was 154/minute.

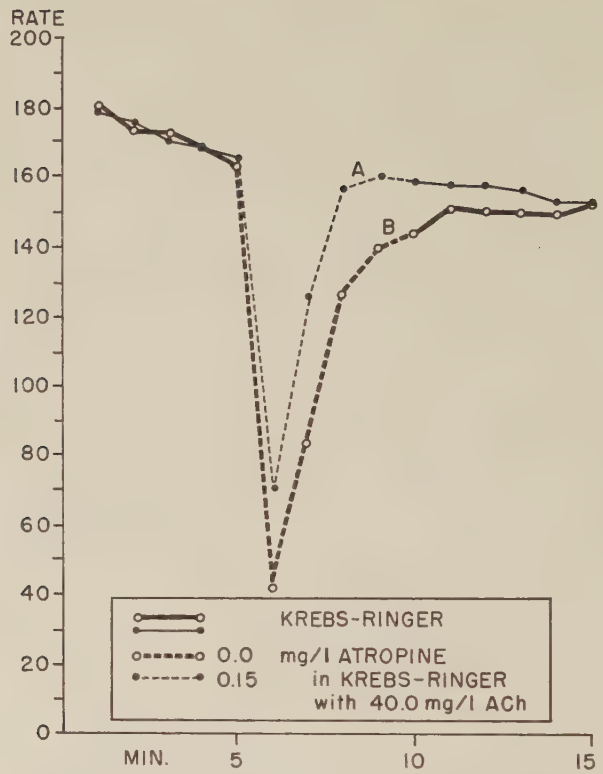

Fig. 1 Graph A: Composite graph to show the activity of 30 hearts in Krebs-Ringer containing 0.15 mg/l of atropine and 40.0 mg/l of ACh. (The lowest rate in each minute for each heart was used in this and the following graphs.) Graph B: Composite graph to show the activity of 30 "concurrent ACh control" hearts in Krebs-Ringer containing 40.0 mg/l of ACh.

II. With the use of Atropine previous to the Atropine-ACh solution

A. Atropine Sulphate at 0.15 mg/l

Thirty hearts were studied individually (graph A of fig. 2). Contraction rates were determined over a 5-minute initial control period; the average of these was 179/minute.

The hearts were then placed for 5 minutes in a 0.15 mg/l solution of atropine in Krebs-Ringer; the deceleration which occurred to an average of 171/minute was not statistically sig-

nificant. The estimated amplitude of contraction in this solution was unchanged from that in the preceding control solution.

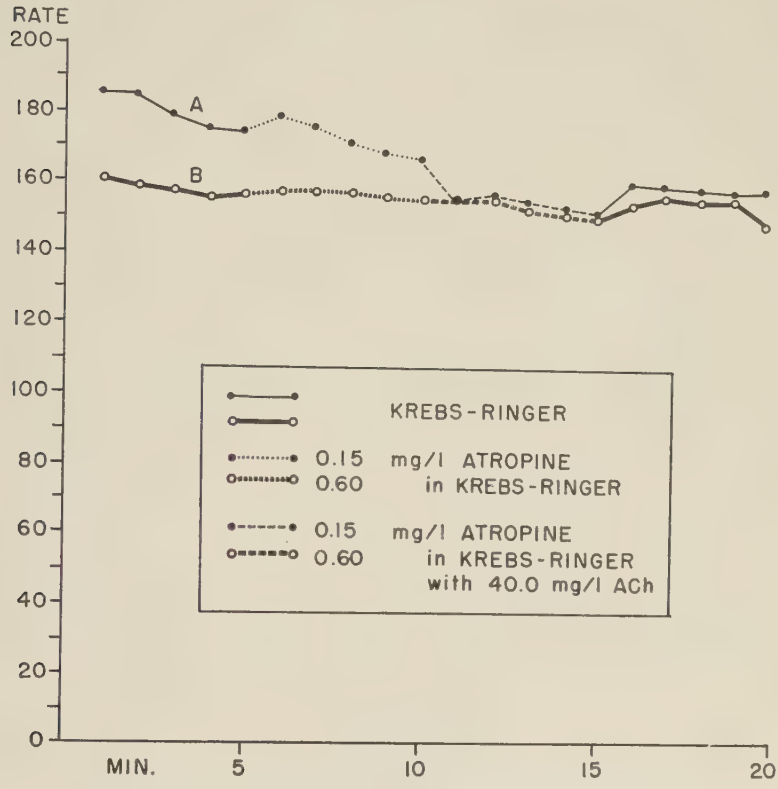

Fig. 2 Graph A: Composite graph to show the activity of 30 hearts in Krebs-Ringer containing 0.15 mg/l of atropine, then in Krebs-Ringer containing in addition 40.0 mg/l of ACh. Graph B: Composite graph to show the activity of 30 hearts in Krebs-Ringer containing 0.60 mg/l of atropine, then in the same solution after the addition of 40.0 mg/l of ACh.

The hearts were then transferred for 5 minutes to Krebs-Ringer containing both atropine (0.15 mg/l) and ACh (40 mg/l). The average rate in this solution was 155/minute, again a change without statistical significance.

Transitory deceleration typical of the ACh effect was not observed in the atropine-ACh solution, and the average of the

lowest rates of the 30 hearts was only slightly less than the average contraction rate (148 vs. 155/minute). Arrest did not occur in the atropine-ACh solution and variations in amplitude of contraction were not observed.

The average terminal control rate was not significantly different from that in the atropine-ACh solution.

B. Atropine Sulphate at 0.60 mg/l

Thirty hearts were studied (graph B of fig. 2), as in the preceding section, except that the concentration of atropine was 0.60 mg/l. A special effort was made to select hearts whose contraction rate had become completely stabilized.

The average initial control rate and also that in the atropine was 158/minute. ACh in solution was injected into the finger bowl containing the atropine solution to avoid disturbing the heart by transferring it, care being taken that thorough mixing occurred. The contraction rate in this solution of the two drugs averaged 154. There were no arrests or marked changes in rate, the average of the lowest rates in the atropine-ACh solutions being 150/minute.

The terminal control rate averaged 156/minute. There is no significant difference between the average rates of the four 5-minute periods during which these hearts were studied. Variations in amplitude were not observed.

III. Experiments to determine the duration of Atropine blocking

Thirty hearts were studied individually. After an initial control period, during which the average rate was 156, the hearts were maintained for 5 minutes in Krebs-Ringer containing 0.15 mg/l of atropine sulphate, where the average rate was 158/minute. The hearts were then transferred to intermediate control solutions without drugs for 15, 30 or 45 minutes (10 hearts each). After this, the hearts were placed in ACh solutions (40 mg/l) for 5 minutes, and finally into terminal control solutions for 5 minutes.

Of the 10 hearts maintained for 45 minutes in the intermediate control, 8 were arrested in the ACh solution for an average of 98 seconds and the remaining two decelerated 27% from the immediately preceding control rates. These effects were comparable to those obtained in the "concurrent ACh control" experiments of Section IV. Less pronounced ACh effects were obtained in the hearts maintained for only 15 and 30 minutes in the intermediate control solutions.

IV. Concurrent ACh controls

Concurrent ACh control observations were made on 30 hearts in the course of the preceding experimentation to test the effectiveness of the ACh solution (graph B of fig. 1).

After each third or fourth experimental heart, a fresh heart was observed during an initial control period (average rate for 30 hearts: 171/minute). This heart was then transferred to a 40 mg/l ACh solution, where 21 of the 30 hearts were arrested within the first minute for an average of 47 seconds. The remaining 9 hearts decelerated to an average of 122/minute, a 29% reduction. The average rate increased during the 5-minute period in the terminal control solution, but recovery was incomplete.

DISCUSSION

It is now the consensus that autonomic drugs act, not upon nerve endings, as was once believed, but directly on effector cells. Some of the most direct evidence for this point of view is derived from experiments with embryonic organs into which the migration of nerve cells and nerve fibers has not occurred.

Few investigators have dealt with the effects of atropine on the embryonic vertebrate heart. Pickering (1893, 1895) obtained typical muscarine and atropine effects only at later (innervated) stages of the chick embryo, whereas at earlier (pre-neural) stages, muscarine had no specific action and atropine had only a comparatively small depressant action. With earlier and later mammalian embryos (ages not given),

however, Pickering (1896) observed typical depression with muscarine, which was removed by the application of atropine.

Fujii ('27) studied the effects of atropine on isolated embryonic chick hearts, and observed inhibition at higher dose levels and stimulation at lower dose levels and at later stages. He stated that the influence of atropine on the vagus nerve endings, like that of ACh, could be demonstrated with certainty only on the fourth day of development, and concluded that autonomic nerves were either entirely undeveloped at earlier stages, or insufficiently developed to be demonstrated by pharmacological methods.

Armstrong ('31, '35) observed atropine blocking of the effects of vagus stimulation and of ACh and pilocarpine on the embryonic fish heart. However, in this heart, ACh was found not to produce its characteristic effects until after innervation, and atropine effects on the pre-neural heart were not demonstrated.

Cullis and Lucas ('36) found that embryonic (pre-neural) chick hearts of less than two days incubation were arrested or markedly slowed by ACh, but there was instant recovery on transference to atropine solution. After preliminary immersion in atropine, ACh effects were reduced or abolished.

Smith, Glassman, Lind, Post, Sohn and Warren ('54) used atropine on older (innervated) embryonic chick hearts, and found that it inhibited cardiac irregularities and acceleration caused by ouabain, but did not of itself cause rate changes.

In the present investigation, it was found that atropine blocking of ACh effects on the embryonic rat heart did not depend on innervation, but could be obtained before invasion of nerve cells and nerve fibers into the organ. The blocking action was thus directly on the heart muscle fiber, and the findings are in accord with those of Cullis and Lucas ('36) on the embryonic chick heart.

Blocking of ACh effects was incomplete when the embryonic rat hearts were transferred directly from a control Krebs-Ringer solution to a solution containing both ACh and atropine. The blocking was complete if the hearts had been in an

atropine solution just previously. Atropine effects on the embryonic rat heart were found to persist for 30 minutes, but after 45 minutes in control solutions the hearts showed ACh effects that were comparable to those obtained in the "concurrent ACh control" experiments of section IV.

In contrast to the results of Pickering (1893, 1895) and of Fujii ('27) on the embryonic chick heart, atropine was found neither to stimulate nor to inhibit rate in the embryonic rat heart. Chronotropic effects of atropine have been denied by other authors: Wedd and Blair ('45) found that atropine in concentrations as high as 1/25,000 had no significant effect on the rhythmicity or contractility of the adult turtle heart. Greiner and Garb ('50) reported no demonstrable effects on irritability or automaticity of cat papillary muscle. Inotropic effects of atropine were not observed in the present investigation.

It is interesting to note that the molar concentration of ACh of the experiments here reported was more than 1,000 times that of the lower atropine concentration (0.15 mg/l). The atropine concentrations used were not much greater than the minimum toxic dose in man (about 0.03–0.05 mg/kg, Berger and Ballinger, '47), and were within the range used with human subjects (as high as 0.7 mg/kg, Cullumbine, McKee and Creasey, '55). Very much higher dose levels of atropine have been used in the rat (25 mg/kg, Tønnesen, '48).

SUMMARY

In summary, it may be stated that under these experimental conditions atropine did not affect heart rate or estimated amplitude, that atropine in low concentrations blocked ACh effects on (pre-neural) embryonic rat hearts if allowed to act on these hearts just before their exposure to ACh, and that the direct action of atropine in blocking ACh effects on embryonic cardiac muscle is therefore indicated.

LITERATURE CITED

ARMSTRONG, P. B. 1931 Functional reactions in the embryonic heart accompanying the ingrowth and development of the vagus innervation. J. Exp. Zool., *58:* 43–67.

———— 1935 The role of the nerves in the action of acetylcholine on the embryonic heart. J. Physiol., *84:* 20–32.

BERGER, A. R., AND J. BALLINGER 1947 The relative toxicity of atropine and novatrine in man. Amer. J. Med. Sci., *214:* 156–158.

CULLIS, W. C., AND C. L. T. LUCAS 1936 Action of acetylcholine on the aneural chick heart. J. Physiol., *86:* 53P–55P.

CULLUMBINE, H., W. H. E. MCKEE AND N. H. CREASEY 1955 The effects of atropine sulphate upon healthy male subjects. Quart. J. Exp. Physiol., *40:* 309–319.

FUJII, M. 1927 Pharmakologische Untersuchungen an embryonalen Hühnerherzen verschiedener Entwicklungsstufen. I. Mitt. Folia Pharm. Jap., *4:* 309–330. (Cited from: Ber. ges. Physiol. u. exp. Pharmakol., *41:* 281, 1927.)

GREINER, T. H., AND S. GARB 1950 The influence of drugs on the irritability and automaticity of heart muscle. J. Pharmacol., *98:* 215–223.

HALL, E. K. 1951 Intrinsic contractility in the embryonic rat heart. Anat. Rec., *111:* 381–399.

———— 1954 Further experiments on the intrinsic contractility of the embryonic rat heart. Ibid., *118:* 175–184.

———— 1955 Arrest and recovery of the embryonic rat heart in mineral acid acidosis. J. Exp. Zool., *129:* 343–356.

———— 1957 Acetylcholine and epinephrine effects on the embryonic rat heart. J. Cell. and Comp. Physiol., *49:* 187–200.

HOGG, I. D. 1957 Developing innervation of the heart in the albino rat. Anat. Rec., *127:* 470.

PICKERING, J. W. 1893 Observations on the physiology of the embryonic heart. J. Physiol., *14:* 383–466.

———— 1895 Further experiments on the embryonic heart. Ibid., *18:* 470–483.

———— 1896 Experiments on the hearts of mammalian and chick-embryos, with special reference to action of electric currents. Ibid., *20:* 165–222.

SMITH, J. A., M. GLASSMAN, A. H. LIND, M. POST, H. SOHN AND S. WARREN 1954 Effects of ouabain on beat and oxygen consumption of embryonic chick hearts. Proc. Soc. Exp. Biol. Med., *86:* 747–749.

TASHIRO, S., C. C. SMITH, E. BADGER AND E. KEZUR 1940 Chromodacryorrhea, a new criterion for biological assay of acetylcholine. Ibid., *44:* 658–661.

TØNNESEN, M. 1948 The absorption and distribution of atropine in rats. Acta Pharmacol. et Toxicol., *4:* 367–378.

WEDD, A. M., AND H. A. BLAIR 1945 Action of atropine on the turtle heart. Proc. Soc. Exp. Biol. Med., *60:* 64–66.

DOSE RELATIONSHIPS AND OXYGEN DEPENDENCE IN ULTRAVIOLET AND PHOTODYNAMIC HEMOLYSIS [1]

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THREE FIGURES

This paper deals with hemolysis brought about by two different kinds of photochemical reaction. The first of these is forwarded by radiation from the ultraviolet spectrum. It may be schematized by

$$X \xrightarrow{h\nu} X_a \qquad (1)$$

where X is a substrate in the cell which is altered photochemically, the superscript hν indicating that X itself is the light-absorber, or chromophore, which captures the quantum hν. The product of the reaction is X_a, the formation of which leads in some way to hemolysis. The steps in this process are not known. This effect we refer to as ultraviolet hemolysis (cf. Cook, '56).

The second kind of reaction is brought about by visible or ultraviolet radiation through the intermediacy of a photosensitizing dye, usually exogenous. It may be schematized by

$$D + X + O_2 \xrightarrow{h\nu} X_{ox} + D \qquad (2)$$

where D is the dye, which in this case is the chromophore. Again the steps are not clear, but in the course of the reaction

[1] A preliminary report on some of the data included in this paper has been published elsewhere (Blum and Cook, '56).

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the substrate becomes oxidized to X_{ox}. D is not used up, as is indicated by its appearance on the right hand side of the reaction scheme. The effects of this kind of reaction, when occurring in biological systems, are commonly described by the term photodynamic action, and we refer to the particular process examined here as photodynamic hemolysis. (For discussions of such processes, cf. Blum, '41; Blum and Kauzmann, '54; Clare, '56.)

The schemes given above are necessarily vague, since we are not clear as to the nature of the substrate X, which could be more than one substance, e.g., protein and/or lipid, and which is not necessarily the same in the two cases. Similarly, we do not know the nature of the products X_a and X_{ox}, which are probably different. However, both reactions initiate hemolytic processes which appear to be very similar in the two cases.

It will be shown in the first part of this paper that the hemolytic process set off by either one of these reactions follows the same kind of kinetics and the same dose-effect relationships. In the second part, the difference in O_2 dependence, which is to be expected from the reaction schemes above, is examined. Since some of the methods used were the same in both studies, these will be described first.

MATERIALS AND METHODS

Rabbit erythrocytes were used in all the experiments reported here except some dealing with the kinetics of photodynamic hemolysis in which human cells were used. (Other experiments have shown that the relationships are the same in both species; the only quantitative differences found lie in the magnitude of the constants in the derived equations.) Coagulation was prevented with either Na-citrate or heparin. The cells were washed three times, then resuspended in 0.9% NaCl-PO_4 (0.139 M NaCl, 0.0088 M Na_2HPO_4, 0.0014 M NaH_2PO_4; pH = 7.4), to make up the original whole blood volume. In some of the longer experiments 10 γ per milliliter

of chloromycetin (Parke, Davis) were added to the suspension to protect the cells from bacterial hemolysis.

For irradiation the washed cell suspension was diluted 1:400 in NaCl-PO_4 and 1 ml of the dilute suspension placed in a quartz dish one inch in diameter. When the cells from this volume of suspension settle to the bottom of the dish, they form virtually a single layer. As shown in figure 1, the dish containing the cells was placed in a metal chamber provided with a quartz window at the bottom through which the cells could be irradiated, and a glass window at the top through which they could be observed. Water at controlled temperature could be circulated through the walls of the chamber, the experiments being carried out near room temperature. Forty-five minutes were allowed for settling of the cells and their equilibration with the environment. In comparative studies two paired chambers were used.

Hemolysis time was measured from the beginning of irradiation until a sharp-bordered object could be clearly seen through the layer of cells, an end-point which is quite reproducible.

In the study of O_2 dependence the chambers were closed with a gas-tight seal as indicated in figure 1. In such studies commercial nitrogen, washed twice in alkaline pyrogallol and equilibrated with NaCl-PO_4 solution of the concentration used for suspending the cells, was passed through one of the chambers. The paired chamber was aerated with filtered room air similarly equilibrated with NaCl-PO_4. The cells in the nitrogen chamber took on the color of reduced hemoglobin within a few minutes, but equilibration was continued for 45 minutes before irradiation was begun.

In order to study the effects of different regions of the spectrum, we used four types of source, combined with several filters. The spectral emissions of these sources and the transmissions of the filters are shown in figure 3, together with other spectral information pertaining to the discussion. In certain cases columns of gas, either air or N_2, were used as filters. For this purpose we used metal tubes 18 inches long,

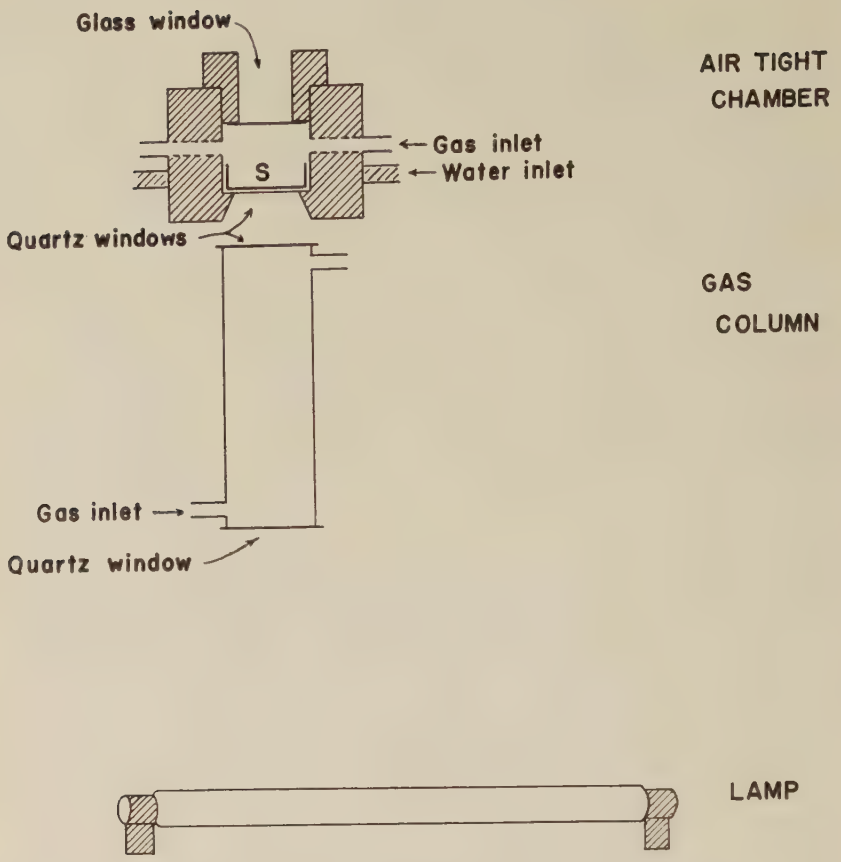


Fig. 1 Diagram of apparatus used for irradiation of red blood cells. Cell suspension placed in quartz dish at S. Further description in text.

blackened inside, and with quartz windows at the ends. The tubes were provided with inlet and outlet (fig. 1) so that the gases could be circulated, thus avoiding the possibility that ozone might accumulate in the air tube. This product of ultraviolet radiation on O_2 also absorbs strongly in the ultraviolet.

Since all the sources were tubular, the same dose-rate [4] for each of the paired chambers could be attained by placing them

[4] The dose-rate may be defined as the number of quanta (or other units of energy) incident per unit time. It thus corresponds to the term *incident intensity*, which was used formerly.

at corresponding positions near the mid-point of the tube. Further insurance against differences in dose-rate was provided by alternating the experimental conditions (e.g., air or N_2) in the two chambers in successive experiments. The distance between cells and source was adjusted according to the conditions required for the particular experiment. In order to study dose-rate dependence we maintained the distance between cells and source constant and altered the dose-rate by means of neutral filters made up of multiple layers of black nylon netting interposed in such a position that they would not cast patterned shadows on the cells. The measured transmissions of these filters were the same for the different sources used, indicating their spectral neutrality.

The apparatus described above was used in most of the studies reported in this paper, but another method was employed for measuring the dose-hemolysis time relationship for photodynamic hemolysis. Cells from heparinized blood were washed and resuspended as described above. This suspension was diluted 1:200 with 10^{-5} M rose bengal in 0.9% NaCl-PO_4 and stirred for 80 minutes in the dark to allow the cells to take up the dye, near equilibrium being reached at the end of this time (Gilbert and Blum, '41). With stirring continued vigorously, the suspension was then illuminated with a 500-watt photoflood lamp at a distance of 6 inches, a test tube filled with water being interposed to focus the beam and to reduce heating by removing the longer wavelengths. The periods of irradiation ranged from 1.5 to 9 minutes. After irradiation the suspension was returned to the dark and sampled at appropriate intervals thereafter. One milliliter of cell suspension was mixed with 1 ml of 3 $\times$ isotonic $NaNO_2$. The hypertonicity of the nitrite solution prevented further hemolysis during manipulations and its reducing power stopped further photochemical reaction. The sample was then rapidly centrifuged and the hemoglobin concentration in the supernate determined by measuring the optical density at 0.42 μ with a Klett-Summerson colorimeter. From this value the percentage hemolysis could be determined. A similar

sample which had been kept in the dark for 80 minutes was used as a standard for zero hemolysis, and one which had been irradiated beyond the point of complete hemolysis served as the 100% standard. No correction for absorption by the dye is necessary at this wavelength.

Dose-hemolysis time relationships

The dose-squared relationship. It was shown by Cook ('56) that the rate of hemolysis varies as the square of the dose. The doses used in those studies were of short duration compared to the hemolysis times, and, under those conditions, reciprocity between dose-rate and duration of the dose was observed for given doses; that is

$$D = It_r = rD' \qquad (3)$$

where D is the dose, I the dose-rate, t_r the duration of the dose, D′ the effective dose,[5] and r a constant.

Hemolysis time varied inversely as the square of the dose; that is

$$1/t_h = KD^2 = K(It_r)^2 \qquad (4)$$

where t_h is the hemolysis time and K is a constant.

Since photodynamic hemolysis is initiated by a photochemical reaction entirely different from that which initiates ultraviolet hemolysis, it has seemed important to compare the dose relationships in the two cases. The method employed, using rose bengal as the sensitizer, has been described in the preceding section. The measurements are in terms of the hemoglobin released from the cells, from which the number of cells hemolyzed is determined. As in the case of ultraviolet hemolysis, if the percentage hemolysis is plotted on a probability grid (probits), and the time after irradiation on arithmetic coordinates, a straight line is obtained. The slope of this line varies with the dose. Thus either the slope of the hemolysis curve or the time to a given percentage hemolysis

[5] Effective dose may be defined as that portion of the incident dose D which is actually absorbed by the chromophore.

may be used to relate hemolysis time to dose. The details of the analysis are given elsewhere (Cook, '56).

Reciprocity having already been established for photodynamic hemolysis (Blum and Gilbert, '40), it was not necessary to repeat this part of the experiment.

TABLE 1

Dose-slope relationships in photodynamic hemolysis

RATIO OF DOSES	RATIO OF SLOPES OF HEMOLYSIS CURVES	EXPONENT OF D $\left(\frac{\text{Log slope ratio}}{\text{Log dose ratio}}\right)$
2.0	4.81	2.3
1.5	2.04	1.8
2.0	3.44	1.8
3.0	7.03	1.8
2.0	3.41	1.8
2.0	7.46	2.9
4.0	25.4	2.3
2.0	4.8	2.3
2.0	4.9	2.3
4.0	23.1	2.3
2.0	3.4	1.8
2.0	5.03	2.3
4.0	17.1	2.05
		Av. 2.1 ± 0.3

For these experiments the probit of percentage hemolysis vs. time after irradiation was plotted for two doses, and the slopes compared. The logarithm of the slope ratio divided by the logarithm of the dose ratio gives their exponential relationship. Table 1 shows that the exponent of D obtained by such comparisons is close to the expected value of 2.

It is clear from these experiments that photodynamic hemolysis, like ultraviolet hemolysis, follows essentially a dose-squared relationship. Whatever the cause of this may be, it is indicated that a common denominator for the two types of hemolysis is to be sought somewhere in the hemolytic mechanism rather than in the dissimilar photochemical reactions which initiate this process.

Dose-rate relationships. As indicated above, reciprocity holds for doses that are of short duration compared to the corresponding hemolysis times. If, on the other hand, the dose were continued through the hemolysis time, another relationship would be expected. Under these conditions

$$t_h = t_r \tag{5}$$

and, substituting in (4),

$$1/t_h = K(It_h)^2. \tag{6}$$

Simplifying and rearranging

$$t_h = \frac{1}{K^{1/3} I^{2/3}} \tag{7}$$

Thus, if the dose is continued up to the moment of hemolysis, the hemolysis time should vary inversely as the ⅔ power of the dose-rate. That this condition is reasonably well satisfied is seen by reference to figure 2. The experiments represented there were carried out according to the method described above, which compares hemolysis times at a constant percent hemolysis. An intermediate pressure mercury arc was used as the source, under three sets of conditions: with a 96% silica glass filter (Corning no. 7910) and the cells equilibrated with room air; without filter and the cells equilibrated with room air; and without filter and the cells equilibrated with N_2. The values in figure 2 have been corrected for these three conditions by arbitrary constants which bring them on to a comparable scale. As is seen from the figure, plotting the log t_h vs. log I gives the expected — ⅔ slope.

The demonstration that when the dose-rate is varied, hemolysis time follows the course expected from the dose-squared relationship, not only confirms that relationship under other conditions, but shows that there is no true "latent" or "induction" period.

Comparable experiments with photodynamic hemolysis gave somewhat variable results. Owing to the very low dose-rates obtainable in this system, such experiments lasted up to 50 hours with concurrent changes in cell fragility. This factor, plus a certain increase in dye uptake by the cells during the

experiment (cf. Gilbert and Blum, '41) were probably responsible for the skewed results.

Oxygen dependence of photodynamic and ultraviolet hemolysis

This study began with the observation that hemolysis brought about by radiation from a low pressure mercury arc in quartz is to a certain extent O_2-dependent. This finding appeared to be in conflict with the general observation that effects of ultraviolet radiation are O_2-independent and specifically with earlier determinations for hemolysis (Hasselbach, '09; Eidenow, '30). The discrepancy seems ultimately

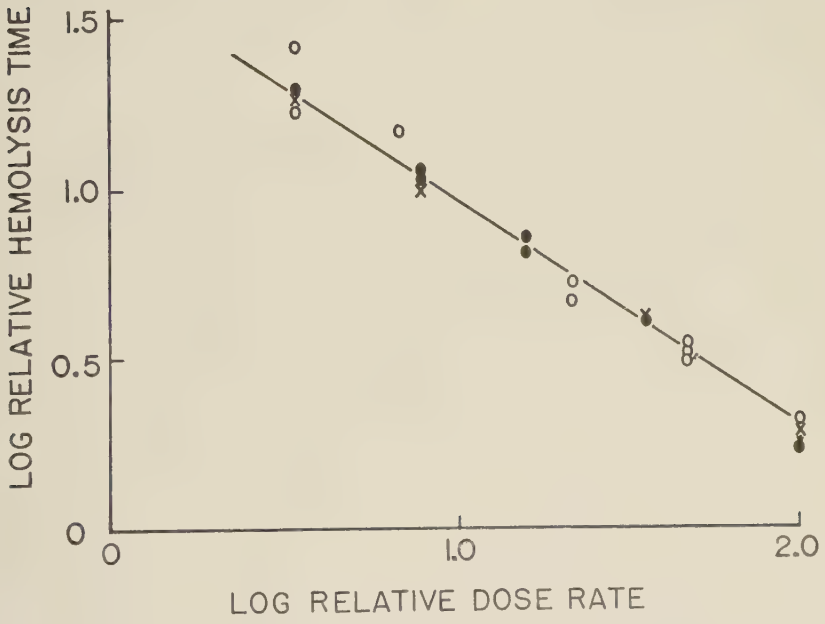


Fig. 2 Hemolysis time (t_h) as a function of dose rate with continuous irradiation, intermediate pressure mercury arc. End points multiplied by arbitrary constants for the type of source and filter combination to bring the points on to the same scale.

Solid line has a slope of $-\frac{2}{3}$. ○, No filter; cells equilibrated with room air; ×, No filter; cells equilibrated with N_2 atmosphere; ●, 96% silica glass filter; cells equilibrated with room air.

explainable in terms of differences in O_2 dependence with different portions of the ultraviolet spectrum, which are described below.

The method we have used compares the hemolysis times for two like samples of red cells, one in air, the other in N_2, when subjected to the same dose-rate of radiation. We may describe the dose-rate hemolysis-time relationship for the two samples according to equation (7):

$$1/t_a = k_a^{1/3} I^{2/3} \tag{8}$$

and

$$1/t_n = k_n^{1/3} I^{2/3} \tag{9}$$

where t_a and t_n are respectively the hemolysis times for the sample in air and for the sample in N_2, and k_a and k_n are respective constants.

Since I has the same value in both cases, we may write

$$(t_a/t_n)^3 = k_n/k_a. \tag{10}$$

The ratio k_n/k_a should measure, formally, the fraction of the total process which is independent of O_2 (as measured between air and N_2 under our experimental conditions). It is seen that the ratio of hemolysis times which we determine experimentally is the cube root of the ratio k_n/k_a. Hence, in table 2, $(t_a/t_n)^3$ is used as the measure of the O_2-independent fraction of the process, and $[1 - (t_a/t_n)^3]$ as the measure of the O_2-dependent fraction. The results are reasonably reproducible for a given condition, but there is a wide variation with the spectral character of the radiation. This requires explanation.

Before attempting such an explanation, let us examine our measure of O_2 dependence a little further. The condition of complete absence of O_2 is, of course, not achievable, but is only approached. It would appear, however, that we have reduced the partial pressure of O_2 below one percent of that of air. The values for O_2 dependence of photodynamic hemolysis given in table 2 are within one percent of the 100% O_2 dependence expected for this process (cf. Spealman and Blum, '37).

The values for k_a and k_n should be determined by at least two major factors: (1) the absorption spectra of the chromophore

Oxygen dependence of hemolysis by radiation from various spectral regions: calculations according to equation (10)

SOURCE	FILTER		t_a	t_n	t_a/t_n	O_2-INDEPENDENT FRACTION $(t_a/t_n)^3$	O_2-DEPENDENT FRACTION $1-(t_a/t_n)^3$
			minutes	*minutes*			
Low-pressure mercury arc	None		45	110	0.41	0.069	
			40	90	0.44	0.085	
			40	95	0.42	0.074	
			25	55	0.45	0.091	
						Av. = 0.080	0.92
	96% Silica glass		31	65	0.48	0.11	
			115	150	0.77	0.46	
			100	150	0.67	0.30	
			105	165	0.64	0.26	
						Av. = 0.28	0.72
Intermediate pressure mercury arc	None		70	70	1.00	1.00	
			115	130	0.88	0.68	
			18	20	0.90	0.73	
			33	36	0.92	0.78	
			16	17	0.94	0.83	
						Av. = 0.81	0.19
	96% Silica glass		45	60	0.75	0.42	
			45	60	0.75	0.42	
			45	60	0.75	0.42	
			45	57	0.79	0.49	
						Av. = 0.44	0.56
Blacklight fluorescent	None		1200	3240	0.37	0.051	
			600	1410	0.43	0.079	
			1080	2520	0.43	0.079	
			840	2355	0.36	0.047	
						Av. = 0.06	0.94
Daylite fluorescent	None	*	20	280	0.071	0.0004	0.9996
		**	105	690	0.152	0.004	
			105	1160	0.091	0.008	
						Av. = 0.006	0.994

* Photosensitized with 10^{-7} M rose bengal.
** Photosensitized with 10^{-8} M rose bengal.

or chromophores, and (2) the photochemical efficiency as affected by the O_2 tension. Thus we might assume that we deal with a single photochemical process, in which case the term k_n/k_a is a measure of the O_2 independence; this is clearly the case in photodynamic hemolysis, where the dye is the chromophore and only the partial pressure of O_2 differs in the two samples. In ultraviolet hemolysis, on the other hand, we might assume that we deal with two separate processes — both leading to hemolysis but having different chromophores — the one process O_2-dependent, the other O_2-independent. In this case, the fraction k_n/k_a should reflect differences in the absorption spectra of the chromophores as well as the O_2 dependence of the one reaction. A third possibility is that we deal with a combination of these things, that is, that there is more than one chromophore and more than one degree of O_2 dependence.[6]

The participation of more than one chromophore in ultraviolet hemolysis most readily explains the variation of O_2 dependence with the spectral character of the radiation, and is reasonable in terms of the chemical makeup of the erythrocyte. There are various reasons for thinking that one of the components concerned in ultraviolet hemolysis is protein

Fig. 3 *Absorption spectra:* Air = transmission of air (Vassy, '41); N_2 is virtually transparent in this region. Pr = protein; the dashed curve represents a protein concentration 50 times that represented by the solid curve. RB, rose bengal; Ppn, protoporphyrin.

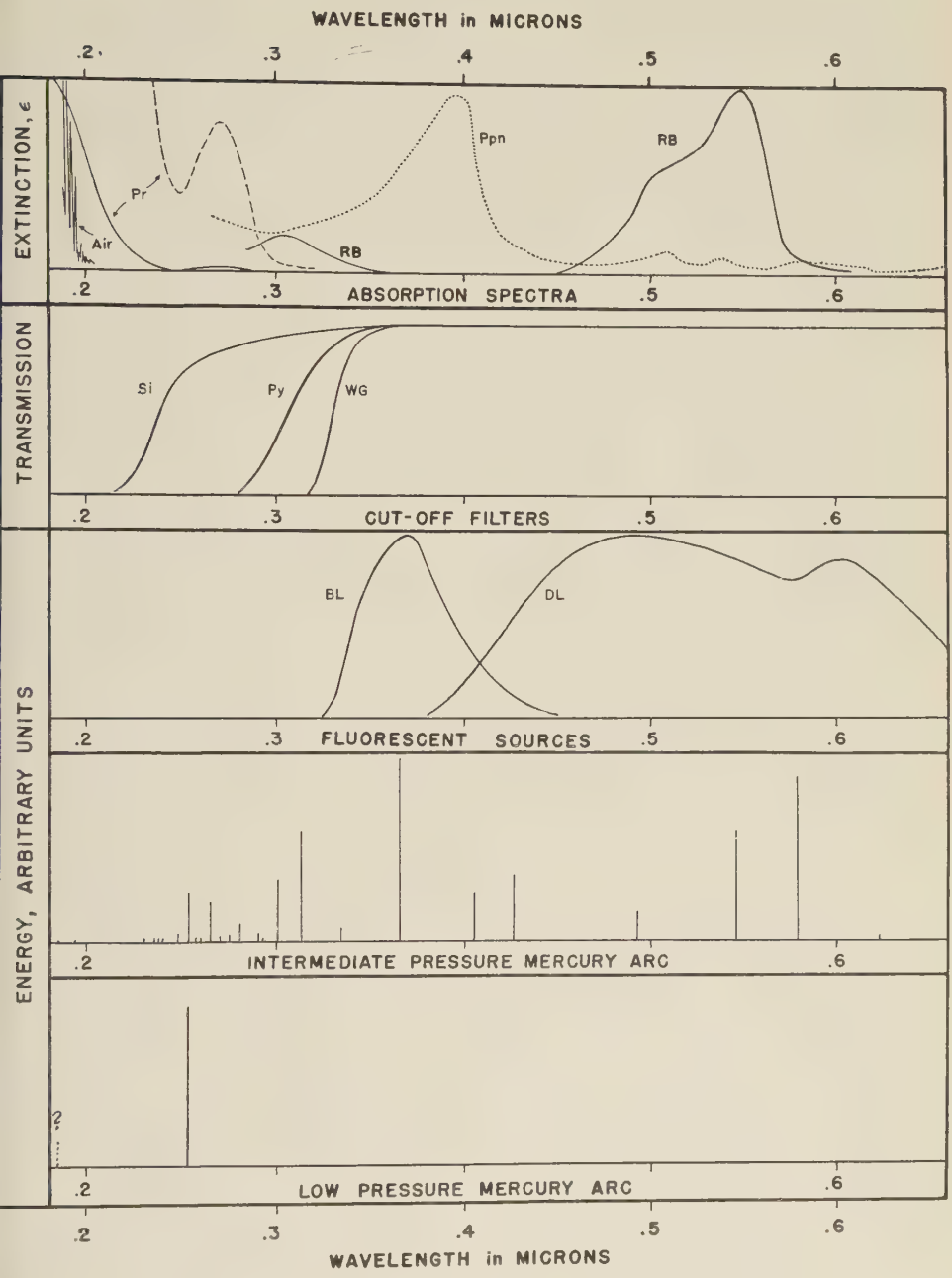
Cut-off filters: Si, 96% silica glass (Corning no. 7910); Py, Pyrex brand glass (Corning no. 7740); WG, window glass.

Fluorescent sources: BL, "blacklight"; DL, "Daylite."

Intermediate pressure mercury arc: A commercial type of arc operating at near atmospheric pressure.

Low pressure mercury arc: This arc was enclosed in a quartz envelope which allowed the 0.185 μ line of mercury to pass. The intensity of this line was not known; it probably constitutes only a few per cent of the total output, but is very effective in producing hemolysis (cf. Cook, '56). The other mercury lines are present in too slight intensity to be represented on this diagram.

[6] In N_2 erythrocytes are slightly swollen and somewhat more susceptible to osmotic hemolysis (Jacobs and Parpart, '31); but this effect is probably trivial in our experiments, and is in opposite direction to the results described here.


WAVELENGTH in MICRONS
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TRANSMISSION
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WG
CUT-OFF FILTERS
ENERGY, ARBITRARY UNITS
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INTERMEDIATE PRESSURE MERCURY ARC
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LOW PRESSURE MERCURY ARC
WAVELENGTH in MICRONS

(Cook, '56) and it is known that photochemical changes in proteins may be independent of O_2 (e.g., McLaren, '49). On the other hand, there is at least one photosensitizing substance present in the erythrocyte in very small quantity, protoporphyrin, and this could be the chromophore for an O_2-dependent part of the process. There may also be traces of other photosensitizers present, e.g., free riboflavin. At very short wavelengths (0.185 μ) O_2 or even H_2O (cf. Repke, '55) may be considered as chromophores.

Let us now examine our data for O_2 dependence of ultraviolet hemolysis (table 2) in terms of these possibilities, using figure 3 and the data of table 3 to assist us in delimiting the spectral regions concerned.

(a) Hemolysis by blacklight radiation. First let us consider the case of hemolysis by the blacklight fluorescent source, which emits radiation between approximately 0.32 μ and 0.45 μ with a maximum near 0.36 μ. We note from figure 3 that this source is rich in wavelengths absorbed by protoporphyrin, and from table 2 that the effect of this radiation is strongly (94%) O_2 dependent. In these experiments, the cells were placed very close to the lamp, which had been encased in a glass jacket containing running water to minimize heating effects.

TABLE 3

Effects of filters on ultraviolet hemolysis by radiation from different Hg arcs

SOURCE	FILTER	t	t_f	t/t_f	FRACTION PASSED BY FILTER $(t/t_f)^3$	FRACTION REMOVED BY FILTER $1 - (t/t_f)^3$
		minutes	*minutes*			
Low-pressure mercury arc	96% Silica glass	40	115	0.35	0.043	
		40	100	0.40	0.064	
		45	105	0.43	0.079	
					Av. = 0.062	0.938
Intermediate pressure mercury arc	96% Silica glass	12	50	0.24	0.014	
		10	45	0.23	0.012	
		11	50	0.22	0.011	
		9	38	0.24	0.014	
					Av. = 0.013	0.987

Even under these conditions, the irradiation had to be very prolonged (a matter of 10 hours or more). The fact that protoporphyrin is present in very small amount may account for this high dosage requirement. Our findings are in agreement with earlier observations by Blum ('41, p. 118) and Meyerstein ('54), who found this effect to be O_2-dependent and attributed it to protoporphyrin.[7] It is possible that the small O_2-independent fraction of hemolysis produced by blacklight (6%) is due to a slight amount of absorption by protein at the lower end of the wavelength range of this source.

(b) Hemolysis by the low pressure mercury arc. Passing to the other extreme of the spectrum, let us examine the effects of the low-pressure mercury arc in quartz, of which the emission may be considered to consist of two lines, 0.185 μ and 0.2537 μ, the latter greatly reduced by the 96% silica filter. Table 3 indicates that about 92% of the hemolytic effect is due to the 0.185 μ line, 8% due to the 0.2537 μ line, this being in general agreement with the estimates of Cook ('56). Table 2 indicates that the combined effect of the two lines is 92% O_2-dependent, whereas the effect of the 0.2537 μ line alone is about 73% O_2-dependent. The latter value may be high because the 96% silica filter no doubt transmits traces of the 0.185 μ line, and a leakage of as little as 6% could account for the total O_2 dependence measured at 0.2537 μ. As will be seen below, the assumption that the O_2 dependence at 0.2537 μ is considerably lower than indicated by the measured value fits better with the findings for the intermediate pressure arc.

We might attribute the high O_2 dependence at 0.185 μ to a photosensitizer absorbing strongly at that wavelength, but while this may be a factor there is at least one other that needs to be considered. Molecular oxygen absorbs to a certain extent at this wavelength and may be considered as a possible chromophore. Spectroscopic measurements with long columns of air (Vassy, '41) indicate a certain amount of absorption

[7] Lepeschkin and Davis ('33) earlier attributed a similar effect to hemoglobin absorption, but Lepeschkin ('31) had found it to be O_2-independent, and it seems likely that their effect was due to heat.

by O_2 at 0.185 μ and very much less at 0.2537 μ, whereas N_2 is completely transparent over this range. Interposing an 18-inch column of air below one sample and a similar column of N_2 below the other, as indicated in figure 1, we found that the air column reduces the hemolytic effect of the low pressure mercury arc to a considerable extent as compared to N_2, but no quantitative comparisons were made. This indicates that a definite amount of the emission from the low pressure arc is absorbed by O_2, presumably principally at 0.185 μ. On the other hand, there is no detectable absorption by air when the intermediate pressure arc is used (table 3) the 0.185 μ emission by that arc being relatively small whereas the 0.2537 μ line is relatively strong. All these findings indicate that O_2 might be a chromophore for hemolysis by wavelength 0.185 μ, but not to any appreciable extent for 0.2537 μ. Wavelength 0.185 μ brings about the formation of ozone from O_2, and this action could possibly result in a good deal of chemical change in the red cell.

There is also a slight amount of absorption by water at 0.185 μ which introduces the possibility of combination with O_2 to form H_2O_2, but it seems unlikely that this could be a very important factor in the hemolytic process. At longer wavelengths it must certainly be negligible.

(c) Hemolysis by radiation from the intermediate pressure mercury arc. Here the problem becomes somewhat more involved since a wider range of wavelengths is concerned. We see from figure 3 that a certain amount of absorption by protoporphyrin, and hence a certain amount of O_2 dependence, is to be expected throughout this range, although the details of the absorption spectrum at shorter wavelengths are missing and only a rough suggestion is given in the region 0.27 to 0.3 μ. It is clear from table 3 that the greater part of the hemolytic effectiveness of this radiation is confined to the shorter wavelengths of the ultraviolet, since the 96% silica filter greatly reduces this effectiveness. It is in these shorter wavelengths that direct action on proteins should be most important, since both the photochemical efficiency (quantum yield) and ab-

sorption increase with decrease in wavelength (Landen, '40; and see Cook, '56). Since this reaction is O_2-independent it might be expected that the O_2 dependency would increase with reduction of the shorter wavelengths by the filter, as is seen from table 2 to be the case. On the other hand, the measurements with the low pressure mercury arc with the 96% silica glass filter indicate a relatively high O_2 dependence (0.28) at .2537 μ. Unless this is to be explained by leakage of the filter as suggested above, the situation is somewhat difficult to rationalize, since the O_2 dependence measured for the intermediate pressure mercury arc is only 0.19.

There is too much information missing for us to overemphasize such quantitative evaluations. They have been introduced to indicate the strong probablity that the partial O_2 dependence of ultraviolet hemolysis may be explained in terms of separate O_2-dependent and independent reactions, including a combination of the two types schematized in equations (1) and (2).

DISCUSSION

One important thing to come out of this study is recognition of the need for understanding the basic dose relationships before estimating the O_2 dependency of any system. Thus, for example, if the ratio t_a/t_n were taken directly as the index — as might seem reasonable in the absence of knowledge of the dose-hemolysis time relationships — one would estimate that photodynamic hemolysis was reduced only to 7 to 18% by placing the cells in an atmosphere of N_2. He might conclude that the partial pressure of O_2 had not been reduced to a low level, or that there was an O_2 independent factor in the process (a conclusion reached by Oster and McLaren ('50) with regard to photosensitized inactivation of tobacco mosaic virus). But when $(t_a/t_n)^3$ is used as the index, it is seen that the N_2 atmosphere has reduced the photochemical reaction underlying hemolysis to less than 1% of that found in air, which indicates that we have reduced the partial pressure of O_2 to that extent. This seems much more reasonable in view of the

experimental conditions and of what is known about the kinetics of photosensitized oxidation.

At the other end of the scale we see that the O_2 dependence for the intermediate pressure mercury arc without filter appears to be only about 8% when the index $1 - t_a/t_n$ is used, but rises to 19% when the cube of the ratio is used. The small difference found on the basis of direct comparison (t_a/t_n) probably accounts for the earlier failures to observe the O_2 dependence of ultraviolet hemolysis with the intermediate pressure mercury arc.

The system studied here may seem a rather special one, yet similar effects of radiation on membranes or interphases in other cells might be expected. In more complex cells, the relatively simple picture presented by hemolysis would be complicated by a variety of factors, and lysis in such cells may be quite complex (e.g., Blum, Cook, and Loos, '54). Although lysis probably does not enter as a factor of any importance in those types of lethal effects most commonly studied, e.g., in survival curves of microorganisms, it is conceivable that it might complicate the picture in some cases, and particularly at high doses because of the dose-squared relationship.

Whether the somewhat complex picture of O_2 dependence found in hemolysis is paralleled elsewhere in photobiologic effects on living systems remains to be established.

SUMMARY

The dose-squared relationship previously reported by one of us (Cook, '56) for the rate of ultraviolet hemolysis has been found to hold under a variety of conditions and to apply to photodynamic hemolysis as well. Since there are several basically different photochemical reactions involved, the common denominator seems to lie in the hemolytic process itself.

Dependence of ultraviolet hemolysis on O_2 is found to vary with the spectral character of the radiation. The explanation is offered that hemolysis is initiated in part by photochemical

reactions that are O_2-independent, e.g., direct alteration of protein; and in part by photochemical reactions that are O_2-dependent, e.g., photosensitization by a naturally occurring porphyrin or other photosensitizing pigment and, in addition, possibly by direct activation of O_2 at very short wavelengths (0.185 μ). The variation in O_2 dependence is thus to be explained on the basis of differences in absorption spectra of the chromophores for these different reactions.

Implications of these findings are discussed.

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LITERATURE CITED

Blum, H. F. 1941 Photodynamic Action and Diseases Caused by Light. Reinhold Publishing Corp., New York.

Blum, H. F., and J. S. Cook 1956 O_2 dependence of hemolysis by ultraviolet light. Fed. Proc., *15:* 20.

Blum, H. F., J. S. Cook and G. M. Loos 1954 A comparison of five effects of ultraviolet light on the Arbacia egg. J. Gen. Physiol., *37:* 313.

Blum, H. F., and H. W. Gilbert 1940 Studies of photodynamic hemolysis with monochromatic light: the reciprocity law. J. Cell. and Comp. Physiol., *15:* 75.

Blum, H. F., and E. F. Kauzmann 1954 Photodynamic hemolysis at low temperatures. J. Gen. Physiol., *37:* 301.

Clare, N. T. 1956 Photodynamic action and its pathological effects. In: Radiation Biology, Vol. III. Ed. by A. Hollaender. McGraw-Hill Book Co., Inc., New York.

Cook, J. S. 1956 Some characteristics of hemolysis by ultraviolet light. J. Cell. and Comp. Physiol., *47:* 55.

Eidinow, A. 1930 The irradiation of the blood in vitro. J. Path. Bact., *33:* 769.

Gilbert, H. W., and H. F. Blum 1942 The mechanism of uptake of the dye, rose bengal, by the red cell. J. Cell. and Comp. Physiol., *19:* 257.

Hasselbach, K. A. 1909 Untersuchungen über die Wirkung des Lichtes auf Blutfarbstoffe und rote Blutkörperchen wie auch über optische Sensibilisation für diese Lichtwirkungen. Biochem. Z., *19:* 435.

Jacobs, M. H., and A. K. Parpart 1931 Osmotic properties of the erythrocyte. II. The influence of pH, temperature and oxygen tension on hemolysis by hypotonic solutions. Biol. Bull., *60:* 95.

LANDEN, E. W. 1940 Quantum yield as a function of wavelength for the inactivation of urease. J. Am. Chem. Soc., *62:* 2465.

LEPESCHKIN, W. W. 1931 Hemolysis and changes in resistance of erythrocytes produced by light. Protoplasma, *14:* 11.

LEPESCHKIN, W. W., AND G. E. DAVIS 1933 Hemolysis and the solar spectrum. Ibid., *20:* 189.

MCLAREN, A. D. 1949 Photochemistry of enzymes, proteins, and viruses. Advances in Enzymol., *9:* 75.

MEYERSTEIN, W. 1954 Photographic phenomena with blood films. J. Phot. Sci., *2:* 174.

OSTER, G., AND A. D. MCLAREN 1950 The ultraviolet light and photosensitized inactivation of tobacco mosaic virus. J. Gen. Physiol., *33:* 215.

REPKE, K. 1955 Uber die Schutzwirkung von Glutathion, Cystein, und Dimercaptopropanol bei der Hämolyse durch ultraviolette Strahlen. Arch. exper. Path. u. Pharmakol., *224:* 300.

SPEALMAN, C. R., AND H. F. BLUM 1937 The photooxidative aspect of photodynamic action. Univ. Calif. Publ. Physiol., *8:* 147.

VASSY, A. 1941 Sur l'absorption atmosphérique dans l'ultraviolet. Ann. Phys., Ser. 11, *16:* 145

ALKALINE DENATURATION AND OXYGEN EQUILIBRIUM OF ANNELID HEMOGLOBINS

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FOUR FIGURES

Various extracellular annelid hemoglobins are very similar with regard to molecular weight, amino acid composition, solubility, and isoelectric point (reviewed: Lemberg and Legge, '49; Prosser, '50; Eliassen, '53). It is reasonable to wonder if this similarity extends to other biochemical properties. A study of the oxygen equilibrium and the alkaline denaturation of two extracellular annelid hemoglobins has been undertaken. The two species used were: (1) the earthworm *Lumbricus terrestris* L., a familiar oligochaete; and, (2) an intertidal terebellid polychaete tentatively identified as *Eupolymnia crescentis* Chamberlin. In addition, alkaline denaturation studies were performed on other terebellid hemoglobins.

MATERIAL AND METHODS

Eupolymnia and other terebellids (*Neoamphitrite* and *Terebella*) were collected intertidally by overturning rocks and were kept in running sea-water a few days before bleeding. *Lumbricus* were collected late on damp nights when the earthworms were partly out of their burrows. Annelids were chilled, cut open along the dorsal surface, and pinned out in a wax-bottomed pan. A small beaker was placed immediately

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adjacent to the anterior part of the dorsal blood vessel, which was then cut. Blood slowly drained into the beaker, a process facilitated by tilting the pan; there was no visible contamination from other body fluids or tissues.

Oxygen dissociation curves were evaluated exactly as in a previous study (Manwell, '58). Blood was diluted with potassium phosphate buffer ($\Gamma/2 = 0.40$); 1:2 for *Lumbricus* hemoglobin and 1:4 for *Eupolymnia*. This brought the hemoglobin concentrations to approximately the same level, 1.5–2.0%. All data were obtained at 10°C, a value well within the normal physiological range of both species of annelids. Data were analyzed by the standard transformation based on the Hill equation; from this the constants "p_{50}" and "n" were obtained (Manwell, '58). "p_{50}" is the partial pressure of oxygen at which there are equal quantities of "reduced" and oxygenated forms of the respiratory pigment; "n" is a measure of the heme-heme interactions and, thus, determines the shape of the oxygen dissociation curve. A convenient physiological measure of the Bohr effect is "φ" $= \Delta \log p_{50}/\Delta$ pH evaluated at pH 7.0–7.5 (Allen and Wyman, '52a).

Alkaline denaturation curves were obtained by spectrophotometrically following the reaction that ensues when a small quantity of hemoglobin is placed in 5 cm^3 of a sodium phosphate buffer, pH 10.5–13.0, $\Gamma/2 = 2.0$ (Manwell, '57). The data are presented as logarithm of the per cent undenatured hemoglobin as a function of time for obvious kinetic reasons.

RESULTS

Oxygen-hemoglobin equilibrium

Oxygen dissociation curves of the blood of these two different annelids are displayed in figures 1 and 2. Although the studies were made under approximately identical conditions, several differences in the properties of the oxygen-hemoglobin equilibrium are immediately obvious:

Lumbricus hemoglobin has: (1) a moderate Bohr effect $-\phi = -0.25$; (2) a high oxygen affinity — $p_{50} = 3.5$–4.8 mm

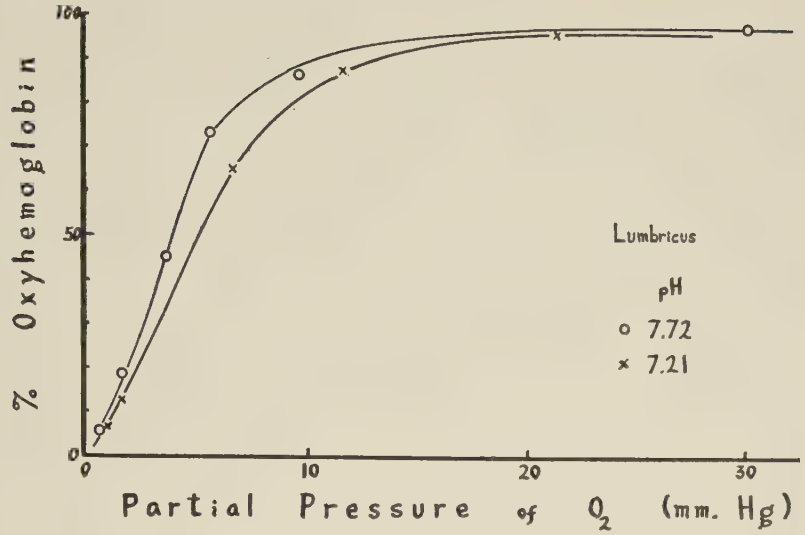

Fig. 1 Oxygen dissociation curves of *Lumbricus* hemoglobin. Blood diluted 1:2 with potassium phosphate buffer of appropriate pH; $\Gamma/2 = 0.40$; 1.5–2% hemoglobin concentration; 10°C.

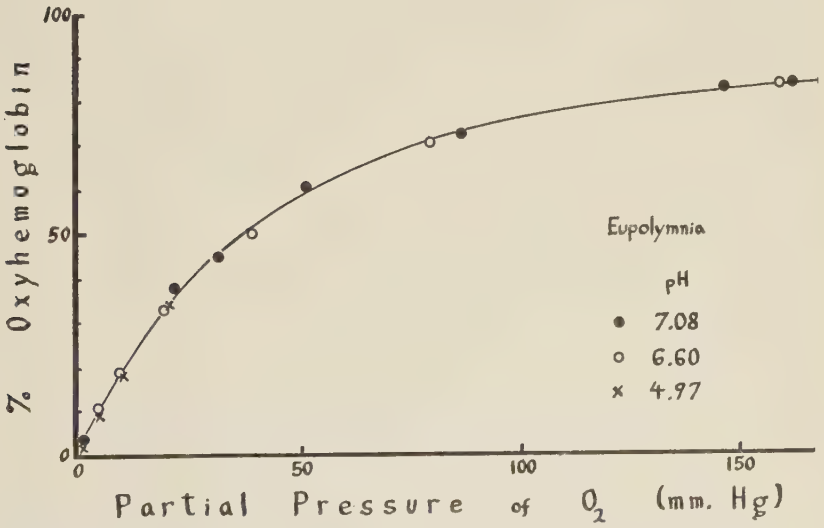

Fig. 2 Oxygen dissociation curves of *Eupolymnia* hemoglobin. Blood diluted 1:4 with potassium phosphate buffer of appropriate pH; $\Gamma/2 = 0.40$; 1.5–2.0% hemoglobin concentration; 10°C.

Hg; and, (3) definite heme-heme interaction — i.e., the hemoglobin possesses a sigmoid oxygen dissociation curve; n = 1.81–1.84 over most of the range in values of "per cent oxyhemoglobin," although "n" approaches one at very low oxygen saturation and approaches 4–5 as the pigment becomes completely converted to oxyhemoglobin.

To the contrary, *Eupolymnia* hemoglobin has: (1) no Bohr effect — $\phi = 0.0$; (2) a very low oxygen affinity — $p_{50} =$ 36 mm Hg; and, (3) almost no heme-heme interaction — i.e., it possesses an essentially hyperbolic oxygen dissociation curve, n being equal to 1.06.

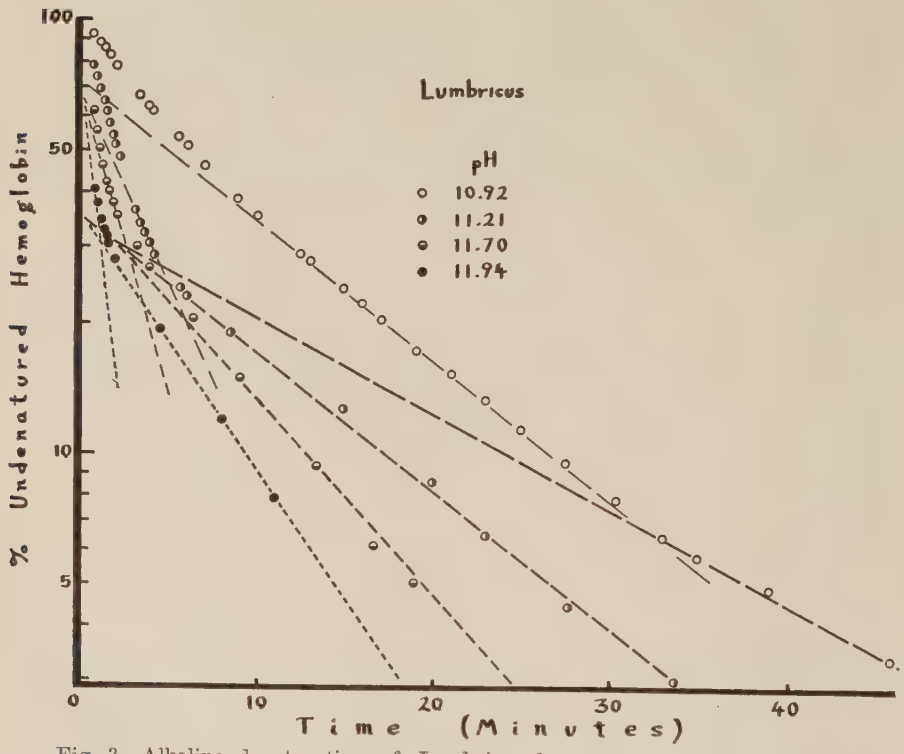

Fig. 3 Alkaline denaturation of *Lumbricus* hemoglobin. Sodium phosphate buffer of appropriate pH; $\Gamma/2 = 2.0$; 24–25°C. *Note:* lines have not been drawn through points indicating the first — fast — alkaline denaturing phase so that the lines indicating the extrapolation of the second and third phases would be more clearly visible.

Alkaline denaturation

Alkaline denaturation curves of *Lumbricus* hemoglobin are displayed in figure 3; those for the terebellids *Neoamphitrite* and *Eupolymnia* are shown in figure 4. Several differences exist in the denaturation behavior of earthworm and terebellid hemoglobins:

Lumbricus hemoglobin denatures triphasically. The proportion of the three unimolecular phases is approximately

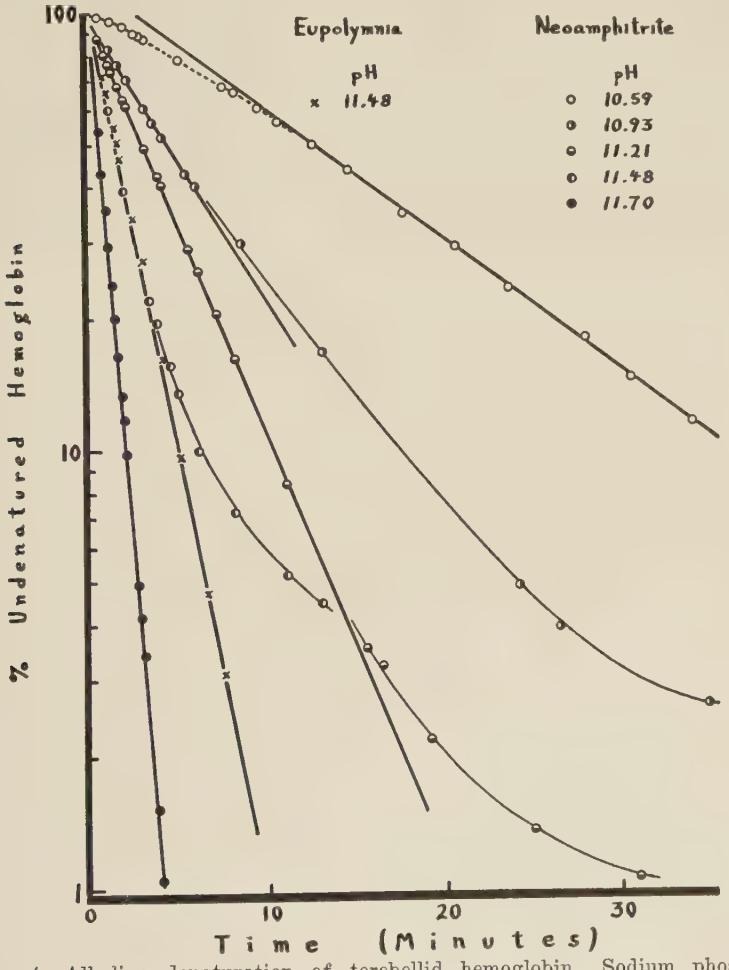

Fig. 4 Alkaline denaturation of terebellid hemoglobin. Sodium phosphate buffer of appropriate pH; $\Gamma/2 = 2.0$; 24–25°C.

1:1:1. (This can be seen by noting that the slowest — third — phase of the alkaline denaturation curves extrapolates to approximately 33% and that the intermediate — second — phase extrapolates to approximately 67%.) The proportions of these phases seems to be independent of the pH of the denaturing medium.

To the contrary, terebellid (*Neoamphitrite, Eupolymnia,* and *Terebella;* experiments on the latter are not included in figure 4) hemoglobins denatures: (1) entirely according to first order kinetics at the most alkaline pH's; (2) with irregular inhibition of an initially first order process at intermediate alkaline pH's; and, (3) with kinetics that cannot be described by any sum of unimolecularly denaturing components at least alkaline pH's but which must represent a sequential with an initial rate-limiting step that must be completed before a first order denaturing phase is begun.

DISCUSSION

Biochemical

Alkaline denaturation. The pH invariance of the proportions of the three unimolecular phases in the denaturation of *Lumbricus* hemoglobin is in contrast to observations on the triphasic alkaline denaturation of the myoglobin and hemoglobin of the horse (Rossi-Fanelli, Azzone, and Mondovi, '55) and on the diphasic denaturation of the hemoglobin of *Cucumaria,* elasmobranchs, teleosts, the garter snake, and the rat (Manwell, '55–'57, unpublished experiments). Evidence for each of three main theories concerning the alkaline denaturation of human hemoglobin can be found in figure 4 — if one selects the proper pH: (1) Human hemoglobin has been claimed to denature unimolecularly under all (Haurowitz, Hardin, and Dicks, '54) or some (Jonxis, '49) experimental conditions; terebellid hemoglobin denatures unimolecularly at the most alkaline pH's. (2) An irregular inhibition of the denaturation reaction has been observed for human hemoglobin and its various derivatives and has been claimed to

represent the effects of CO_2 and reaction products (Baar and Hickmans, '41); the three alkaline denaturation curves of *Neoamphitrite* hemoglobin at intermediate alkaline pH's show this irregular inhibition. (3) That the denaturation reaction of adult human hemoglobin is not unimolecular but some kind of a sequential process has been shown by Betke ('52, '54); clearly, *Neoamphitrite* hemoglobin at pH 10.59 would support this theory.

Diphasic alkaline denaturation curves have been repeatedly interpreted as evidence for the normal existence of two kinds of adult human — or other mammalian (rat and rabbit) — hemoglobin (Brinkman and Jonxis, '35, '36; Ramsey, '41; Jonxis, '49; Schapira, Dreyfus, and Kruh, '51; Haurowitz, Hardin, and Dicks, '54), despite lack of agreement between some of the workers and arguments to the contrary (Baar and Hickmans, '41; Betke, '54). The experiments on terebellid hemoglobins indicate that there appears to be "heterogeneity" only at intermediate alkaline pH's. On the other hand, the constancy of the proportions of the three alkaline denaturing phases of *Lumbricus* hemoglobin could indicate the normal existence of three different biochemical "species" of hemoglobin in that form. However, that the three "components" are present in approximately equal quantities is rather surprising. One could as well postulate negative heme-heme interactions and sequential denaturation; under such conditions one would expect a simple ratio in the proportions of the denaturing phases. It is interesting that in the oxygen equilibrium interactions between oxygen-affine groups (heme-heme interactions), and between oxygen-affine and proton-affine groups (Bohr effect) occur in *Lumbricus* — but not *Eupolymnia* — hemoglobin. *Eupolymnia* — and other terebellid — follows a very different denaturation pattern, which appears to be basically unimolecular. A non-specific inhibition occurs at certain pH's and an initial lag occurs at the lowest pH's; however, neither of these indicate definite interaction between hemes in the denaturation process.

Physiological and ecological

Studies on *Arenicola marina* and *A. cristata* (Barcroft and Barcroft, '24; Wolvekamp and Vreede, '40; Allen and Wyman, '52b; Manwell, '57, unpublished experiments), which like *Eupolymnia* are intertidal polychaetes, indicate that the respiratory properties of their hemoglobin are similar to those of *Lumbricus* hemoglobin. *Arenicola* hemoglobin has large heme-heme interactions, a high oxygen affinity, and a moderate Bohr effect.

Jones' ('54) investigations on the two different hemoglobins (one in the coelom, one in the blood vessels) of the errant polychaete *Nephthys hombergii* reveal certain similarities to the situation in *Eupolymnia* in that there is very little heme-heme interaction (for both *Nephthys* hemoglobins n = 1.0–1.2). However, the oxygen affinities of both pigments are much higher (p_{50} = 5.5–7.5 mm Hg at 15°C) than that of *Eupolymnia* hemoglobin. *Nephthys hombergii* vascular hemoglobin has a slight "normal" Bohr effect (calculated from Jones' data, $\phi = -0.18$), whereas the coelomic hemoglobin has a definite "reverse" Bohr effect at physiological pH's ($\phi = +0.36$).

The high affinity of, and the presence of heme-heme interactions and a "normal" Bohr effect in, *Lumbricus* and *Arenicola* hemoglobins would enable these respiratory pigments to function in oxygen transport under conditions of low internal oxygen tensions. Such low "arterial" and even lower "venous" oxygen tensions would be expected (1) under conditions of low ambient oxygen tension or (2) as a result of an inefficient organ of external respiration. The following information indicates that the first condition is typical of *Arenicola* and the second, of *Lumbricus:*

Arenicola has a definite L- or U-shaped burrow; the oxygen tension in the water contained therein is low (13 mm Hg; Jones, '54) but sufficient to keep the blood completely oxygenated even throughout low tide (Eliassen, '55) due to the combination of the extensive parapodial gills functioning as

an efficient organ of external respiration and the high oxygen affinity of the hemoglobin (*Arenicola cristata* hemoglobin is 95% oxygenated at 3 mm Hg at 12°C; yet, so sigmoid is the oxygen dissociation curve that the pigment is only 4% oxygenated at 0.5 mm Hg — Manwell, '57, unpublished experiments).

Lumbricus, to the contrary, is exposed to oxygen tensions near that of air; however, this form has a respiratory barrier consisting of an unspecialized surface of cuticle, epidermis, and hypodermis. One would expect that the hemoglobin of *Lumbricus* would not be of much significance in oxygen transport at low *ambient* oxygen tensions, for there would not be a sufficient gradient of oxygen tension to load the hemoglobin with oxygen. Hence, one would predict that carbon monoxide would have a proportionately greater effect in diminishing respiration at high rather than low ambient oxygen tensions; this is exactly what has been observed (Krüger, '38, '40; Johnson, '41). This is true, however, only up to a certain point; one might also expect that at high oxygen tensions the effect of carbon monoxide might be reduced because of an appreciable amount of oxygen carried in physical solution; such an effect has been observed at low temperature (9°C) where a combination of increased O_2 solubility and decreased metabolic rate favored the demonstration of this compensation effect (Krüger and Becker, '40). Decrease in the extent of inhibition of *Tubifex* respiration by CO at low oxygen tensions (< 25 mm Hg) has been observed by Dausend ('31) and at high oxygen tensions (> 120 mm Hg) by Krüger ('55a); hence, the hemoglobin of this aquatic oligochaete is also functioning in oxygen transport with a considerable gradient in oxygen tension across the epithelium. On the basis of CO-poisoning experiments the idea concerning the function of hemoglobin in *Lumbricus* and *Tubifex* is very likely true of other terrestrial (Mendes and Valente, '53) and aquatic (Mendes, Perez-Gonzalez, and Coutinho, '51) oligochaetes.

The lack of evolution of a specialized organ of respiration in *Lumbricus* and other oligochaetes could represent (1) phy-

logenetic inability or (2) the result of a definite selection pressure in favor of *low internal* oxygen tensions. The latter alternative represents a novel approach to the function of respiratory pigments: to maintain a suitable supply of oxygen to the tissues but at a low activity (partial pressure) of that gas. The idea of protection of the tissues from high oxygen tensions rests on — or provides an explanation of — several different studies: (1) Fox and Taylor ('55) have shown that such hemoglobin-containing forms as *Tubifex, Planorbis,* and *Chironomus* survive better and grow faster in water equilibrated with 4% oxygen than in water equilibrated with air. The hemoglobin-containing pulmonate *Planorbis* was found to be much more sensitive to "oxygen-poisoning" than its non-hemoglobin-containing relative *Limnaea.* (2) Yet, *Planorbis,* specialized in the production of vascular hemoglobin, requires only half as much oxygen per unit of body weight as the unspecialized *Limnaea* (Krüger, '55a). Krüger ('55b) has also shown that the oxygen consumption rate of non-hemoglobin-containing oligochaetes is greater than that of hemoglobin-containing oligochaetes. (3) Exposure of some invertebrates — *Arenicola, Tubifex,* and adult *Planorbis* — to low oxygen tensions for prolonged periods of time fails to induce an increase in hemoglobin concentration (Fox, '55). (4) Such an assumption of avoidance of "oxygen-poisoning" will explain anomalous effects — or lack of effects — with CO-poisoning of certain hemoglobin-containing invertebrates (Lindroth, '42; Prosser, '50; and Krüger, '55b).

In contrast to the situation in *Arenicola* the hemoglobin of *Nephthys* is probably non-functional when the tide goes out (Jones, '54). *Eupolymnia* hemoglobin with its extremely low oxygen affinity would be even less able to function in oxygen transport when the tide is out. In this terebellid the oxygen transport features a large range in internal oxygen tensions. The profuse branchiae at the anterior end of *Eupolymnia* and other terebellids present a large thin-walled surface for the diffusion of gases. These branchiae are normally waved about in the sea-water, the remainder of the worm being located in

a tube under or along side of a partially imbedded rock. As peristaltic contractions travel along the body of terebellids, there is undoubtedly ventilation of the burrow. Movement of water in the burrow is probably also provided by the activities of the large commensal scale-worm that is often found with terebellids. Observations on living terebellids indicate that the blood is often the bright red color of oxyhemoglobin; the oxygen dissociation curve in figure 2 indicates that this would be possible only at relatively high internal oxygen tensions (100 mm Hg). Chlorocruorin of *Sabella (Spirographis) spallanzanii* resembles *Eupolymnia* hemoglobin in that it has a low oxygen affinity ($p_{50} = 30$ mm Hg at 17°C: Fox, '32). Experiments with CO-poisoning of chlorocruorin-containing worms indicate that the pigment is especially functional at high oxygen tensions, including that of air (Ewer and Fox, '40; Mendes, '50). Hence, it is reasonable to assume that high oxygen tensions, or at least a high "arterial" oxygen tension, are maintained in *Sabella;* it is very significant that *Sabella* is not only resistant to "oxygen-poisoning" at 100% O_2 but also actually survives better at this high oxygen tension than at that of air (Fox and Taylor, '55). Hence, such intertidal polychaetes as *Eupolymnia* and *Sabella* are specialized for oxygen transport under conditions of high internal oxygen tension — in marked contrast to *Arenicola, Lubricus,* and *Tubifex.* Undoubtedly intermediate conditions exist with regard to blood gas transport in the Annelida.

It can be concluded that there are great differences in the oxygen equilibrium of various annelid hemoglobins and that these differences appear to have evolved in phylogenetic response to the different selection pressures of different environments and different respiratory physiologies.

SUMMARY

1. The oxygen equilibrium and alkaline denaturation behavior of earthworm *(Lumbricus)* and terebellid polychaete

(Eupolymnia and *Neoamphitrite)* hemoglobins have been determined.

2. Earthworm and terebellid hemoglobins have very different alkaline denaturation curves. Each of three theories regarding the kinetics of the alkaline denaturation reaction and heterogeneity or homogeneity of normal adult human hemoglobin is supported by experiments on terebellid hemoglobin — at the proper pH. *Lumbricus* hemoglobin is most unusual in that the proportions of the three separate denaturing phases are approximately equal and invariant of pH change.

3. *Lumbricus* hemoglobin, with its high oxygen affinity, heme-heme interactions, and moderate "normal" Bohr effect, appears to be specialized for oxygen transport under conditions of low internal oxygen tensions; in this way it resembles *Arenicola* hemoglobin.

4. *Eupolymnia* hemoglobin has an extremely low oxygen affinity, no heme-heme interactions, and no Bohr effect; this hemoglobin would be expected to function in an oxygen transport involving high "arterial" oxygen tensions — like *Sabella* chlorocruorin.

5. It has been postulated on evidence based on CO-poisoning, survival and growth at different oxygen tensions, failure of low oxygen tensions to evoke increased hemoglobin production, and decreased metabolic rate of certain hemoglobin-containing forms over their non-hemoglobin-containing relatives, that in some — *but not all* — invertebrates the function of the respiratory pigment is to allow oxygen transport without exposing the tissues to high oxygen tensions — i.e., to avoid "oxygen-poisoning."

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LITERATURE CITED

Allen, D. W., and J. Wyman 1952a The oxygen equilibrium of duck hemoglobin. J. Cell. and Comp. Physiol., *39:* 391–394.

———— 1952b The oxygen equilibrium of hemerythrin of *Arenicola cristata.* Ibid., *39:* 383–389.

Baar, H. S., and E. M. Hickmans 1941 Alkali denaturation of oxyhaemoglobin, haemoglobin, carbonmonoxyhaemoglobin, methaemoglobin, and cyanmethaemoglobin. J. Physiol., *100:* 3P–4P.

Barcroft, J., and H. Barcroft 1924 The blood pigment of Arenicola. Proc. Roy. Soc., *96B:* 28–42.

Betke, K. 1952 Zur Frage der Hämoglobinfractionen des Erwachsenenblutes. Naturwissenschaften, *39:* 308–309.

———— 1954 Der Menschliche Rote Blutfarbstoff bei Fetus und Reifem Organismus. Springer-Verlag, Berlin, pp. 41–50.

Brinkman, R., and J. H. P. Jonxis 1935 The occurrence of several kinds of haemoglobin in human blood. J. Physiol., *85:* 117–127.

———— 1936 Alkaline resistance and spreading velocity of foetal and adult types of mammalian haemoglobins. Ibid., *88:* 162–166.

Dausend, K. 1931 Über die Atmung der Tubificiden. Zschr. vergl. Physiol., *14:* 557–608.

Eliassen, E. 1953 The physiology of the vascular system of invertebrates. I. A monography on the blood pigments. Univ. Bergen Årbok, Naturvit. rekke, *11:* 1–65.

———— 1955 The oxygen supply during ebb of *Arenicola marina* in the Danish Waddensea. Ibid., *12:* 1–9.

Ewer, D. W., and H. M. Fox 1940 On the function of chlorocruorin. Proc. Roy. Soc., *129B:* 137–153.

Fox, H. M. 1932 The oxygen affinity of chlorocruorin. Ibid., *111B:* 356–363.

———— 1955 The effect of oxygen on the concentration of haem in invertebrates. Ibid., *143B:* 203–214.

Fox, H. M., and A. E. R. Taylor 1955 The tolerance of oxygen by aquatic invertebrates. Ibid., *143B:* 214–225.

Haurowitz, F., R. L. Hardin and M. Dicks 1954 Denaturation of hemoglobins by alkali. J. Phys. Chem., *58:* 103–105.

Johnson, M. L. 1941 The respiratory function of the haemoglobin of the earthworm. J. Exp. Biol., *18:* 266–277.

Jones, J. D. 1954 Observations on the respiratory physiology and on the haemoglobin of the polychaete genus *Nephthys,* with special reference to *N. hombergii* (Aud. et M.-Edw.) Ibid., *32:* 110–125.

Jonxis, J. H. P. 1949 Foetal haemoglobin and Rh antagonisms. In: Haemoglobin. Ed. F. J. W. Roughton and J. C. Kendrew. Buttersworth, London, pp. 261–268.

KRÜGER, F. 1938 Neue Versuche über die Bedeutung des Haemoglobins im Blute des Regenwurmes. Verhandl. der Deutschen Zoolog. Gesellschaft, *1938:* 141–146.

———— 1940 Beiträge zur Physiologie des Haemoglobins wirbelloser Tiere. I. Versuche über die Atmung normaler und mit Kohlenoxyd vergifteter Regenwürmer bei Temperaturen um 20°C. Zschr. vergl. Physiol., *28:* 43–66.

———— 1955a Hämoglobinvorkommen bei Wirbellosen. Verhandl. der Deutschen Zoolog. Gesellschaft, *1955:* 81–96.

———— 1955b Beiträge zur Physiologie des Hämoglobins wirbelloser Tiere. III. Zur vergleichenden Atmungsphysiologie der Enchytraiden. Zschr. vergl. Physiol., *37:* 118–127.

KRÜGER, F., AND H. BECKER 1940 Beiträge zur Physiologie des Hämoglobins wirbelloser Tiere. II. Vergleich der Atmung normaler und mit Kohlenoxyd vergifteter Regenwürmer bei einer Temperatur von 9°C. Ibid., *28:* 180–196.

LEMBERG, R., AND J. W. LEGGE 1949 Hematin Compounds and Bile Pigments. Interscience, New York, p. 749.

LINDROTH, A. 1943 Die biologische Bedeutung des "Hämoglobins" (Erythrocruorins) der Wirbellosen. Ergebnisse der Biologie, *19:* 324–374.

———— 1957 Alkaline denaturation of hemoglobin of adult and postlarval *Scorpaenichthys marmoratus.* Science, *126:* 1175–1176.

MANWELL, C. 1958 Oxygen equilibrium of the hemocyanin and myoglobin of the amphineuran mollusc *Cryptochiton stelleri* Middendorf. J. Cell. and Comp. Physiol., *52:* 341–352.

MENDES, E. G. 1950 On the respiratory function of chlorocruorin. Pubbl. Staz. Zool. Napoli, *22:* 340–367.

MENDES, E. G., M. D. PEREZ-GONZALEZ AND M. L. COUTINHO 1951 On the function of haemoglobin in limnic oligochaeta. Bol. Fac. Fil. Cien. Letr. Univ. S. Paulo Zoologia, *16:* 289–302.

MENDES, E. G., AND D. VALENTE 1953 The respiratory metabolism of tropical earthworms. I. The respiratory rate and the action of carbon monoxide at normal oxygen pressure. Ibid., *18:* 91–102.

PROSSER, C. L. 1950 Comparative Animal Physiology. Saunders, Philadelphia, pp. 290–340.

RAMSEY, H. J. 1941 A comparative study of hemoglobin denaturation. J. Cell. and Comp. Physiol., *18:* 369–377.

ROSSI-FANELLI, A., G. F. AZZONE AND B. MONDOVI 1955 Alkali denaturation of myoglobin and hemoglobin. Studies on the kinetics of the reaction. Arch. Biochem. Biophys., *58:* 119–123.

SCHAPIRA, G., J. C. DREYFUS AND J. KRUH 1951 Recherches sur la biochimie de l'hémoglobine à l'aide du fer radioactif. Bull. Soc. Chim. Biol., *33:* 812–814.

WOLVEKAMP, H. P., AND M. C. VREEDE 1940 On the gas binding properties of the blood of the lugworm *(Arenicola marina* L.*).* Arch. néerl. physiol., *25:* 265–276.

OXYGEN EQUILIBRIUM OF *CUCUMARIA MINIATA* HEMOGLOBIN AND THE ABSENCE OF THE BOHR EFFECT

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ONE FIGURE

In contrast to the remainder of the Echinodermata, in which there is but a single instance of the occurrence of a respiratory pigment (Hyman, '55), hemoglobin occurs in erythrocytes in representatives of at least three of the 5 orders of holothurians: *Cucumaria miniata* (Crescitelli, '45), *C. frauenfeldi* (Hogben and van der Lingen, '28), and *Thyone briareus* (Svedberg, '33) in the Dendrochirota; *Paracaudina chilensis* and *Molpadia roretzii* (Kobayashii, '32), and *Caudina sp.* and *Molpadia arenicola* (the author) in the Molpadonia; and, *Stichopus californicus* (Prosser and Judson, '52) in the Aspidochirota. In the last-named example the hemoglobin is restricted to the blood vessels (lacunar system); however, in *Cucumaria miniata* and the various molpadids hemoglobin occurs in the water-vascular system and the blood vessels as well as in the perivisceral coelom. Spectral properties of the hemoglobin from the blood vessels and the perivisceral coelom of *Paracaudina chilensis* are identical (Kobayashii, '32), although the red blood cell count of and the cell types found in these body fluid compartments are different (Kawamoto, '27). Most studies on sea cucumber hemoglobins have been limited to spectral properties.

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There appears to be but one published oxygen dissociation curve of a holothurian hemoglobin — at unknown pH: the perivisceral hemoglobin of *Paracaudina chilensis* (Kawamoto, '28). He found a sigmoid coefficient, "n," equal to 0.821. Such a value of $n < 1$ is most unusual and would indicate the presence of *negative* heme-heme interactions. Clearly, a more extensive investigation of the oxygen equilibrium of various sea cucumber hemoglobins is warranted.

MATERIAL AND METHODS

Cucumaria miniata, a large (10–30 cm long) sea cucumber, is found intertidally under rocks on San Juan Island. Specimens were kept in running sea-water until bleeding. This was accomplished by slicing through an interambulacral area of the body wall of an unanesthesized animal. The perivisceral fluid was allowed to flow through a piece of plastic screen in order to remove any of the very fragile visceral elements that might have broken loose. The erythrocytes, which comprise up to 10% of the perivisceral fluid, are immeshed in a weak clot that was broken up by stirring. Clotting did not seem affected by citrate or heparin. Erythrocyte-containing fluid from the water-vascular system (collected from the Polian vesicles) was found in many — but not all — cases not to clot. Erythrocytes were washed twice in large volumes of isotonic sodium chloride and then hemolyzed 1:9 in distilled water: in a few instances the amount of water was decreased to 1:4. Stromata were removed by prolonged centrifugation at 0–1°C. The hemoglobin solution was used immediately for determination of the oxygen dissociation curve by a previously described technique (Manwell, '58a). Hemoglobin solutions were diluted with an equal volume of potassium phosphate buffer ($\Gamma/2 = 0.40$). The oxygen equilibrium was evaluated at two temperatures, 10 and 26°C; the former is the physiological one.

RESULTS

Typical oxygen dissociation curves of *Cucumaria miniata* hemoglobin at two different temperatures and at various pH's

are shown in figure 1. The constants "p_{50}" and "n" that fit these and additional data to the Hill approximation (Manwell, '58a) are shown in table 1. The following conclusions are possible: (1) At 10 or 26°C there is no Bohr effect — at least at physiologically potential pH's. (2) n averages 1.3–1.4 under all conditions, except the most alkaline at which it approaches, but does not become less than, one. (3) A heat of oxygenation can be calculated through the use of an inte-

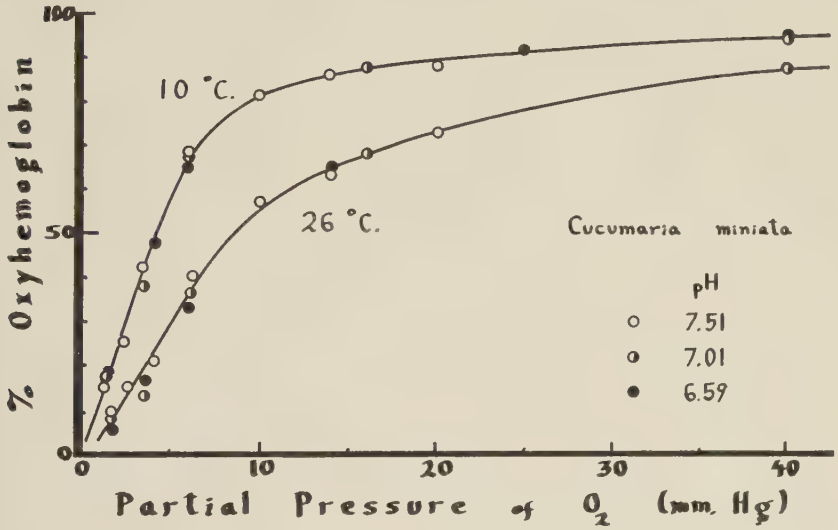

Fig. 1 Oxygen equilibrium of *Cucumaria miniata* hemoglobin. 1.5% hemoglobin solutions in potassium phosphate buffer; final ionic strength, 0.2.

grated form of the van't Hoff equation, defining the equilibrium constant as the reciprocal of "p_{50}" (Glasstone, '47); the value is — 8.4 kcal.

It is difficult to obtain data much below pH 6, for the hemoglobin then rapidly forms a flocculent precipitate in contrast to other invertebrate and vertebrate hemoglobins.

A few preliminary experiments were performed on erythrocyte suspensions at 10°C. The result of these is shown in table 1 and indicates that the absence of the Bohr effect and the small positive heme-heme interactions are unchanged by

TABLE 1

The oxygen equilibrium of cucumaria miniata hemoglobin

pH	n	p_{50}	NUMBER OF DETERMINATIONS
		mm Hg	
	Hemoglobin solutions — 10.0–10.8°C		
8.13	1.04	2.36	4
7.98	1.28	2.37	6
7.51	1.34	3.84	7
7.01	1.33	3.85	5
6.59	1.34	3.51	5
5.93	1.30	3.63	4
	Hemoglobin solutions — 25.5–26.5°C		
8.13	1.17	6.12	4
7.98	1.25	5.98	6
7.50	1.28	8.20	7
7.01	1.30	9.02	5
6.59	1.47	9.44	5
	Erythrocyte suspensions — 9.8–10.1°C		
9.48	1.47	10.5	8
7.81	1.46	11.6	9
7.44	1.50	12.5	7
6.04	1.46	8.0	8

the erythrocyte. The difference in oxygen affinity (measured by p_{50}) is probably the result of the difference in hemoglobin concentrations, 30% in the cell, 1.5% in the hemoglobin solutions—a dilution effect (Hill and Wolvekamp, '36).

DISCUSSION

Biochemical

The data in table 1 indicate that n is never less than one. That it approaches one at the most alkaline pH's resembles studies of Riggs ('52) and could likewise represent the oxidation of -SH groups. One experiment performed on the blood of a molpadid tentatively identified as *Caudina sp.* resulted in a value of $n = 2.0$. Hence, the *negative* heme-heme interactions indicated by Kawamoto's ('28) value of $n = 0.821$ for *Paracaudina* hemoglobin do not exist in either of these other two holothurian hemoglobins.

Not only is the nature of the oxygen equilibrium of holothurian hemoglobins similar to that of other hemoglobins, but the difference in the absorption spectra noted by Kobayashii ('32) may be a relative than an absolute one. Kobayashii had found that *Paracaudina chilensis* and *Molpadia roretzii* hemoglobins have approximately 10% higher absorption at the β band than at the α band. However, the author has observed that, although this increase in optical extinction is as high as 15% for *Molpadia arenicola* oxyhemoglobin, it is only 3% for *Caudina sp.* oxyhemoglobin. In addition, Crescitelli's ('45) absorption spectra for *Cucumaria miniata* oxyhemoglobin indicate that absorption at both maxima is of identical intensity — as is approximately the case for other sufficiently studied hemoglobins. It is interesting to note that, while Crescitelli ('45) found a different heme respiratory pigment in *Molpadia intermedia, Molpadia roretzii* and *M. arenicola* have hemoglobin.

Although extremely variable values of the heat of oxygenation of hemoglobin have been observed (reviewed: Paul and Roughton, '51), their value for sheep hemoglobin, –8.2 kcal., is very close to that observed for *Cucumaria miniata* hemoglobin, –8.4 kcal. Theoretical considerations on the nature of the oxygen-metal bond in respiratory pigments argue for a reasonable constancy in the value of the heat of oxygenation — suitably corrected for contributions from heme-linked groups (Klotz and Klotz, '55). Values of –8.8 and –9.3 kcal. for adult and fetal spiny dogfish hemoglobins have been observed (based on data in Manwell, '58b). Therefore, in this respect also holothurian and other hemoglobins are similar.

Physiological

The absence of a Bohr effect has been considered a primitive characteristic associated with the absence of a circulatory system (Florkin, '49); this statement was based on the absence of interaction between proton-affine and oxygen-affine centers in the hemerythrin of the sipunculids *Sipunculus nudus* and

Golfingia (formerly *Phascolosoma*) *sp.,* and in the hemoglobin of the echiuroid *Urechis caupo,* although McCutcheon ('36) had already shown the absence of a Bohr effect in bullfrog tadpole hemoglobin. Recent studies by the author have indicated the absence of a Bohr effect in the hemerythrins of *Phascolosoma agassizii* (Manwell, '58c) and *Dendrostomum zostericolum* (unpublished); the hemocyanins of the amphineurans *Cryptochiton stelleri* (Manwell, '58a) and *Stenoplax* (*Ischnochiton*) *conspicua* (unpublished); and, the hemoglobins of the polychaete *Eupolymnia* (Manwell, '59) and the holothurian *Cucumaria miniata.* Clearly, the condition is more common than has been suspected and is associated strictly with neither the absence of a circulatory system nor a primitive phylogenetic position.

McCutcheon ('36) explained the absence of the Bohr effect in tadpole hemoglobin as being correlated with the importance of the loading tension — rather than the unloading tension — of oxygen in the blood. If the venous oxygen tension is always low, then a "normal" Bohr effect would not be necessary to facilitate the unloading of oxygen to the tissues. When the ambient oxygen tension becomes low, there is usually a concomittant rise in the CO_2 tension; under such conditions a "normal" Bohr effect could result in a shifting of the oxygen dissociation curve so far to the right that the blood would not become sufficiently saturated with oxygen in the gills. This explanation is also reasonable for the previously enumerated invertebrates, all of which have two things in common: (1) They are not noted for periods of great activity. (2) They are frequently found intertidally and, thus, occasionally meet temporary situations in which there is a decrease in available O_2 and a rise in CO_2.

Redmond ('55) has shown that very low oxygen tensions are characteristic of the blood of a number of decapod crustaceans. While the forms that he studied have a Bohr effect, no significant change in pH between arterial and venous blood was detected; hence, the Bohr effect is of no importance in blood gas transport in these crustaceans — in contrast to various

cephalopods in which the large Bohr effect accounts for up to one-third of the oxygen given up to the tissues (Wolvekamp, '49). The various chitons studied by the author resemble the crustaceans in having (1) a low concentration of hemocyanin and (2) the blood so low in oxygen saturation as to appear almost colorless when samples are withdrawn. A very steep oxygen gradient exists across the gill membranes in both these crustaceans and amphineurans.

The critical oxygen tension (reviewed: Prosser, '50) of *Urechis caupo* is 100 mm Hg. The author, using a polarographic respirometer based on the technique of Baumberger ('39), found that for *Cryptochiton stelleri* the critical oxygen tension is 80–90 mm Hg and for *Cucumaria miniata* it is 100–115 mm Hg; these were the highest critical oxygen tensions obtained with this particular technique. While the critical oxygen tension depends in part on the method used to determine oxygen consumption rate at different oxygen tensions, it is important to note that these values are high for invertebrates possessing well-defined critical oxygen tensions, which have been evaluated by a variety of techniques (Prosser, '50). The closeness of the critical oxygen tensions of *Urechis, Cryptochiton,* and *Cucumaria* to the oxygen tension of sea-water (130–150 mm Hg) suggests that there is little compensatory ability in the aerobic aspects of respiration in these three "Bohr effect-less" animals.

In conclusion, absence of a Bohr effect has been evolved polyphyletically and represents: (1) a situation phylogenetically tolerated in an organism which does not require large variations in the extent of aerobic metabolism, and/or (2) the results of selection pressure against a normal Bohr effect when this would interfere with the loading of sufficient oxygen by the respiratory pigment and yet not be needed to facilitate release of oxygen to the tissues.

SUMMARY

Oxygen dissociation curves of *Cucumaria miniata* hemoglobin have been obtained under a variety of conditions. The

hemoglobin lacks a Bohr effect. The heat of oxygenation and the nature of the oxygen equilibrium of sea cucumber hemoglobin are similar to those of other hemoglobins. Previously reported spectral differences between holothurian and other hemoglobins are not significant for all holothurian hemoglobins.

The absence of a Bohr effect is a property of several hemoglobins, hemocyanins, and hemerythrins. The physiological aspects of this phenomenon are discussed.

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LITERATURE CITED

Baumberger, J. P. 1939 The relation between the "oxidation-reduction potential" and the oxygen consumption rate of yeast cell suspensions. Cold Spring Harbor Symp., *7:* 195–213.

Crescitelli, F. 1945 A note on the absorption spectra of the blood of *Eudistylia gigantea* and of the pigment in the red corpuscles of *Cucumaria miniata* and *Molpadia intermedia.* Biol. Bull., *88:* 30–36.

Florkin, M. 1949 Biochemical Evolution. Transl. and ed. by S. Morgulis. Academic Press, New York, p. 157.

Glasstone, S. 1947 Thermodynamics for Chemists. Van Nostrand, New York, p. 522.

Hill, R., and H. P. Wolvekamp 1936 The oxygen dissociation curve of haemoglobin in dilute solution. Proc. Roy. Soc., *120B;* 484–495.

Hogben, L., and J. van der Lingen 1928 On the occurrence of haemoglobin and of erythrocytes in the pervisceral fluid of a holothurian. J. Exp. Biol., *5:* 292–294.

Hyman, L. H. 1955 The Invertebrates. Vol. 4, Echinodermata. McGraw-Hill, New York, p. 763.

KAWAMOTO, N. 1927 The anatomy of *Caudina chilensis* (J. Müller) with especial reference to the perivisceral cavity, the blood and the water vascular systems in their relation to blood circulation. Sci. Rep. Tôhoku Univ., series 4 (Biology), *2:* 239–264.

———— 1928 Oxygen capacity of the blood of certain invertebrates which contains haemoglobin. Ibid., *3:* 561–575.

KLOTZ, I. M., AND T. A. KLOTZ 1955 Oxygen-carrying proteins: a comparison of the oxygenation reaction in hemocyanin and hemerythrin with that in hemoglobin. Science, *121:* 477–480.

KOBAYASHII, S. 1932 The spectral properties of haemoglobin in the holothurians, *Caudina chilensis* (J. Müller) and *Molpadia roretzii* (v. Marenzeller). Sci. Rep. Tôhoku Univ., series 4 (Biology), *7:* 211–228.

MCCUTCHEON, F. H. 1936 Hemoglobin function during the life history of the bullfrog. J. Cell. and Comp. Physiol., *8:* 63–81.

MANWELL, C. 1958a Oxygen equilibrium of the hemocyanin and myoglobin of the amphineuran mollusc *Cryptochiton stelleri.* J. Cell. and Comp. Physiol., *52:* 341–352.

———— 1958b A "fetal-maternal shift" in the spiny dogfish *Squalus suckleyi.* Physiol. Zool., *31:* 93–100.

———— 1958c Oxygen equilibrium of *Phascolosoma agassizii* hemerythrin. Science, *127:* 592–593.

———— 1959 Alkaline denaturation and oxygen equilibrium of annelid hemoglobins. J. Cell. and Comp. Physiol., *53:* 61–84.

PAUL, W., AND F. J. W. ROUGHTON 1951 The equilibrium between oxygen and sheep haemoglobin at very low percentage saturations. J. Physiol., *113:* 23–35.

PROSSER, C. L. 1950 Comparative Animal Physiology. Saunders, Philadelphia, pp. 246–247.

PROSSER, C. L., AND C. L. JUDSON 1952 Pharmacology of haemel vessels of *Stichopus californicus.* Biol. Bull., *102:* 249–251.

REDMOND, J. R. 1955 The respiratory function of hemocyanin in crustacea. J. Cell. and Comp. Physiol., *46:* 209–247.

RIGGS, A. F. 1952 Sulfhydryl groups and the interaction between the hemes in hemoglobin. J. Gen. Physiol., *36:* 1–16.

SVEDBURG, T. 1933 Sedimentation constants, molecular weights, and isoelectric points of the respiratory proteins. J. Biol. Chem, *103:* 311–325.

WOLVEKAMP, H. P. 1949 Oxygen and carbon dioxide transport by blood containing haemocyanin. In: Haemoglobin. Ed. by F. J. W. Roughton and J. C. Kendrew. Buttersworth, London, pp. 301–311.

THE INTERACTION BETWEEN SODIUM OUTFLUX AND THE SODIUM TRANSPORT SYSTEM IN THE FROG SKIN [1]

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Quantitative description of the kinetics of active ion movement has usually entailed some use of the diffusion equations. Since these equations are independent of any particular chemical mechanism, they are unlikely to yield information concerning a molecular model of the process. Recently a few attempts have been made to treat ion transport from a different point of view, one which assumes that a chemical reaction involving the ion underlies active transport. Thus, both "carriers" (Kirschner, '55, Kato et al., '56, Snell and Leeman, '57) and "non-carriers" (Patlak, '57) undergo such a reaction.

In addition to the assumption that an ion-carrier complex was formed our treatment included a second hypothesis to account for a phenomenon so far described only for the frog skin. When the outside of an isolated skin is bathed by a solution containing little sodium the sodium outflux (M_o)[2] is small. When the concentration in the external solution is high (e.g., 115 mM/l) M_o is much larger (Kirschner, '55). Thus the magnitude of the flux seems to be conditioned by the composition of the solution *into which* the ions are diffusing.

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[2] The terminology used here is conventional. Outflux is the rate of movement of ions which originate in a solution bathing the *inside* of the skin, move through and appear in a solution bathing the *outside* of the preparation. Influx is the rate of movement in the opposite direction.

To rationalize this phenomenon it was supposed that the outward-diffusing ions pass close to the (inward-oriented) pump, and that if one of them collides with a free carrier molecule it is pumped back to the inside solution. Obviously ions returned to the inside solution contribute nothing to M_o. The dependence of M_o on external sodium concentration can be explained as follows. When the concentration of sodium in the outside solution is low the carrier will exist largely in the free form, and many of the ions diffusing out will be pumped back into the inside solution. Thus, only a fraction of those which start through the skin appear in the outside solution, and the outflux, measured, e.g., with isotopic sodium will be low. But with high Na in the external solution the transport system will be loaded, and there is little probability that an ion deriving from the *inside* solution will collide with an unoccupied carrier. As a result most of the ions which start to move out get through the skin, and M_o will be high. The equation based on this hypothesis was tested, and it proved to be remarkably successful in describing the phenomenon.

The scheme is accessible to experiment in another way. Inhibition of the pump when it is moving sodium inward (high external sodium) should have little effect on M_o because there is little interaction between outflux and carrier in such an experiment. On the other hand, if inhibition is induced when most of the carrier is free M_o should increase, for the low outflux depends on a functional sodium pump. Thus, inhibition of the transport system under two sets of conditions, high and low external sodium concentrations, provides another test of theory.

METHODS

The short-circuited frog skin (Ussing and Zerahn, '52) was used in these experiments. The inside of the skin was bathed by a Ringer's solution the pH of which was 8.2 (except in the low-pH experiments). The same solution was used to bathe the outside of the preparation where a high sodium concentration was desired. For low-sodium experiments a Ringer's solu-

tion was used in which choline replaced the sodium. In our early experiments the solution was completely free from sodium, but later a small quantity of Na-Ringer's was added so that the effect of the inhibitor could be monitored more effectively. The concentration in the bath was 1 mM/l which sufficed to generate a small net transport, but was low enough to leave most of the the pump uncomplexed (Kirschner, '55).

The outflux was measured with Na^{22}. The isotope was added to the inside solution and about an hour allowed for equilibration. Duplicate 0.50-ml aliquots were removed from the outside solution after specified intervals. These were plated, dried, and counted with a conventional end-window GM tube. Flux values in successive control periods were very reproducible. Usually three one-hour control periods were run after which the inhibitor was added. Samples taken subsequently gave values for M_o in the presence of the inhibitor. Thus each preparation served as its own control.

A large number of compounds are known to stop the active movement of sodium by the frog skin. Metabolic inhibitors proved to be unsuitable for reasons which will be discussed below. However, several inhibitors seem to act more directly on the transport system. Thus, anticholinesterases stop sodium transport by decreasing the influx (Kirschner, '53), but have no effect on the oxygen consumption of the skin (unpublished data). Strophanthin-K also inhibits sodium transport although its effects have not been described in detail for the frog skin. The effects of low pH in the inside solution have been described in detail by Schoffniels ('56). Eserine sulfate was added in crystalline form to give a concentration slightly less than 1×10^{-2} M. Strophanthin was added from a stock solution to give a final concentration of 2.5 μg/ml in the inside solution. The pH of the inside solution was lowered to 5 by the addition of glacial acetic acid. Reversibility was tested by adding solid $NaHCO_3$ to bring the pH back to 8.2.

The animals used were *R. pipiens* which had been stored in aquaria at 5°C for several weeks prior to use.

EXPERIMENTAL

Inhibition with high external sodium

When the outside of the skin was bathed with ordinary Ringer's solution the transport mechanism was engaged in moving sodium from the outside to the inside solution. Table 1 shows how the addition of eserine and strophanthin affected the outflux. In both cases the influx dropped to a low value (these data are not shown) indicative of inhibition of the pump, but the outflux changed very little even three hours after addition of the inhibitor. A decrease in pH in the inside solution also causes the influx to drop with no consistent effect on the outflux (Schoffniels, '56).

TABLE 1

The effect of inhibitors on outflux with high external sodium

PERIOD [1]	ESERINE				STROPHANTHIN			
	Control		Inhibitor		Control		Inhibitor	
	Min.	Outflux [2]	Min.	Outflux [2]	Min.	Outflux	Min.	Outflux
1	60	0.031	60	0.027	60	0.061	60	0.078
2	60	0.045	60	0.047	60	0.061	60	0.086
3	60	0.047	60	0.065	60	0.072	60	0.106

[1] Successive periods were run with no interruptions. The inhibitor was added and inhibitor period 1 commenced immediately after the termination of control period 3.

[2] Micromoles per cm^2 per hour.

Inhibition with low external sodium

Table 2 shows data from representative experiments using eserine and strophanthin. The experiment with eserine was run with no sodium in the outside solution, and hence influx was not monitored. In the strophanthin experiment the sodium concentration (1 mM/l) was sufficient to generate a net flux which dropped markedly when the inhibitor was added. In both cases a large increase in outflux was noted in contrast to the results described above for high external sodium.

A series of experiments was run in which the pump was inhibited by lowering the pH of the inside solution to 5. Data

from one of these runs is shown in table 3, and again the rise in outflux during inhibition is apparent. Reversibility of the pH effect was checked simply by neutralizing the acid with an excess of bicarbonate (the addition of $NaHCO_3$ caused a negligible change in specific activity of the isotope). The results are shown in the same table. No attempt was made to test reversibility for the other agents, although it is known that the action of eserine on *net* sodium movement is reversible (Kirschner, '53). The inhibition of transport by strophanthin is also reversible (unpublished experiments).

TABLE 2

The effect of inhibitors on outflux with low external sodium

PERIOD	ESERINE				STROPHANTHIN			
	Control		Inhibitor		Control		Inhibitor	
	Min.	Outflux	Min.	Outflux	Min.	Outflux	Min.	Outflux
1	30	0.025	30	0.043	60	0.037	60	0.067
2	30	0.037	30	0.127	—	—	60	0.329
3	30	0.031	30	0.091	120	0.037	60	0.269

Protocol as in table 1.

TABLE 3

The effect of pH on outflux with low external sodium

CONTROL (pH 8.2)			INHIBITED (pH 5.1)			REVERSAL (pH 8.2)		
Period	Min.	Outflux	Period [1]	Min.	Outflux	Period [2]	Min.	Outflux
1	45	0.078	3	65	0.096	5	60	0.107
2	60	0.071	4	55	0.209	6	60	0.058

[1] Period 3 followed the second control period with no delay.

[2] Period 5 followed 4 without delay.

DISCUSSION

Two points having to do with experimental protocol merit discussion.

It was noted that the action of the inhibitors used is not nearly so consistent in the low sodium experiments as in those with high concentrations. Eserine and low pH sometimes have little effect on sodium movement when choline Ringer's is used.

It was for this reason that the use of a sodium-free solution was abandoned in favor of one which incorporated a low concentration of sodium. The use of sodium did not render the results more consistent, but since net transport could be monitored electrically the experiments in which the inhibitor was ineffective could immediately be distinguished and discarded prior to analysis. No explanation can be offered for the inconsistency under these conditions, but although it might be interesting in another context, it was an undesirable artifact in this work.

In addition, certain compounds which stop sodium transport proved to be unsuitable for this work. Most of the metabolic inhibitors fall into this class, as does strophanthin-K when its concentration exceeds 3–4 μg/ml. These agents cause an increase in outflux at high sodium concentrations, a phenomenon first described by Fuhrman ('52) for inhibition with dinitrophenol. This observation seems to conflict with theory, for inhibition of the transport mechanism should have no effect on outflux at high transport rates. Two recent papers, however, indicate that the action of most of the metabolic inhibitors may be complex. In the first of these it was shown that dinitrophenol not only stops the transport of sodium ion across the skin, but causes the epithelial cells to lose potassium and to gain sodium. Loss of ability to maintain the normal intracellular steady state was not a necessary concomitant of transport inhibition; certain xanthines stopped net movement without disturbing the electrolyte balance of the cells (Levinsky and Sawyer, '53). These observations have recently been extended to include a much larger group of compounds, only three of which (Na-fluoracetate, azide and diethyl malonate) act solely on the transport mechanism. Most of them had the dual effect noted for dinitrophenol (Huf, et al., '57).

This complex action may also include another effect; partial loss of the ability of the skin to act as a diffusion barrier. That is, the treatment makes it more permeable to ions. Thus, we have noted a large decrease in DC resistance after using iodoacetate, strophanthin (5–10 μg/ml), and after prolonged

exposure to dinitrophenol. This was accompanied by a huge increase in the outflux of sodium; a rise much larger than any of those noted here. Thus the apparent bias involved in selecting only certain inhibitors is really a matter of following the dictates of our working hypothesis; implicit in the latter is the condition that the action of a test compound, whatever its mechanism, be restricted to inhibition of the sodium pump. Apparently eserine, hydrogen ion and low concentrations of strophanthin act specifically on transport across the skin. The xanthines and the group of compounds described by Huf would probably be suitable test agents, although they have not been used in this laboratory.

These experiments show that the inhibitors used cause an increase in M_o only when the sodium concentration in the outside solution is low. They have little effect (frequently no effect) at high concentrations. This is exactly what is predicted by theory, and hence the observations provide support for an "in series" arrangement of the outward-moving stream of ions and the carrier system.

SUMMARY

The outflux of sodium ion through the isolated frog skin was measured before and after inhibition of active sodium transport by strophanthin-K, eserine, and low pH. When the transport mechanism was maximally active inhibition was accompanied by at most a small increase in outflux. Inhibition during periods of low activity caused the outflux to rise markedly. The theoretical implications of these observations were described, and some limitations of the experimental procedure discussed.

LITERATURE CITED

FUHRMAN, F. 1952 Inhibition of active sodium transport in the isolated frog skin. Am. J. Physiol., *171:* 266.

HUF, E., N. DOSS AND J. WILLS 1957 Effect of metabolic inhibitors and drugs on ion transport and oxygen consumption in isolated frog skin. J. Gen. Physiol., *41:* 397.

KATO, H., B. ZWOLINSKI AND H. EYRING 1956 J. Physical chem., *60:* 404.

KIRSCHNER, L. B. 1953 Effect of cholinesterase inhibitors and atropine on active sodium transport across frog skin. Nature, *172:* 348.

———— 1955 On the mechanism of active sodium transport across the frog skin. J. Cell. and Comp. Physiol., *45:* 61.

LEVINSKY, N., AND W. SAWYER 1953 Relation of metabolism of frog skin to cellular integrity and electrolyte transfer. J. Gen. Physiol., *36:* 607.

PATLAK, C. 1957 Contributions to the theory of active transport. Bull. Math. Biophys., *19:* 209.

SCHOFFNIELS, E. 1956 Influence du pH sur le transport actif a traverse le peau de grenouille. Arch. Int. Physiol., *63:* 513.

SNELL, F., AND C. LEEMAN 1957 Temperature coefficients of the sodium transport system of isolated frog skin. Biochem. Biophys. Acta, *25:* 311.

USSING, H., AND K. ZERAHN 1951 Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol. Scand., *23:* 110.

OXIDATIVE METABOLISM AND ABSORPTION SPECTRA OF ANAEROBICALLY GROWN YEAST

MANOMETRIC DATA AND ABSOLUTE ABSORPTION SPECTRA

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TWO FIGURES

Ever since Pasteur's work (1876) it has generally been believed that without oxygen yeast can either grow only to a limited extent, or not at all (Brown, '05; Windisch, '32; White and Munns, '51). The slight growth that was obtained occasionally under anaerobiosis was attributed to the traces of oxygen left in the medium. The careful work of Stier and his collaborators (Stier, Scalf and Brockman, '50; Stier, Scalf and Peter, '50; Andreasen and Stier, '53, '54, '56) finally clarified this problem. They showed (1) that under strict anaerobiosis yeast cells stopped growing entirely when carried for several transfers in a synthetic medium containing all the previously known growth factors; (2) that a culture medium containing vegetable oils (e.g., 0.3% wheat germ oil) and high concentrations (6–7%) of yeast extract permitted anaerobic growth to reach the same final cell density, about 400×10^6 cells/ml, as that reached in aerobic cultures; and (3) finally they were able to identify two new growth factors, required for anaerobic growth only, ergosterol and long-chain unsaturated fatty acids (like oleic acid). In a synthetic medium supplemented with minute amounts of these substances, final cell densities of ca. 200×10^6 cells/ml were ob-

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served under anaerobiosis. The fact that this is only about half of the maximum value that can be obtained in the high-yeast extract medium was interpreted by Andreasen and Stier ('56) as indicating a requirement for a third anaerobic growth factor, as yet unidentified.[2]

These findings made it now possible to investigate the metabolism and enzymatic composition of yeast cells that were grown anaerobically in cultures permitting rapid and continuous growth, and therefore could validly be compared with that of aerobically grown cells. The fact that the metabolism of yeast cells can vary to a great extent depending on the culture conditions, particularly on the presence or absence of oxygen, was first indicated by the work of Meyerhof ('25), then by Warburg ('27), Trautwein and Wassermann ('30), as well as numerous other workers since. The connection between cytochrome composition and respiratory rate was shown by Euler, Fink, and Hellström ('27), Fink ('32), and Fink and Berwald ('33), who found the three α bands of cytochromes *a, b,* and *c,* in baker's yeast, but only two α bands, attributed later to cytochromes a_1 and b_1, in brewer's yeast, and maintained that the difference between the two kinds of yeast was caused by the availability of oxygen during growth. This view was supported by their finding that a culture with one type of cytochrome spectrum could be converted into the other by growing it with or without aeration. Borei and Sjöden ('43) measured the extracted cytochrome *c* in yeast cells grown under various conditions of aeration and found that the amount of cytochrome *c* was proportional to the degree of aeration during growth.

In none of these studies were the cells grown under strict anaerobiosis, which accounts for finding some cytochrome *c* in all cultures, neither was the possibility excluded that during growth the composition of the population changed through

[2] Harris ('56) showed that the continuous anaerobic cultivation of yeast in the presence of ergosterol and oleic acid was possible for as many as 173 generations, and that the frequency of respiratory-deficient mutants did not increase in the absence of oxygen (Harris, M.; J. Cell. and Comp. Physiol., *48:* 95–112).

mutation and selection. These objections were met by the studies of Ephrussi and Slonimski ('50), who used adequate techniques to ensure anaerobiosis during the growth of the cultures, and observed the transition from the anaerobic to aerobic cytochrome spectrum under non-growing conditions. Their spectroscopic results confirmed previous observations on brewer's yeast, and established the presence of two pigments in anaerobically grown yeast, called provisionally cytochromes a_1 and b_1, in contrast to aerobically grown yeast which is known to have cytochromes a, a_3, b, and c (review by Smith, '54), as well as cytochrome c_1 (Lindenmayer and Estabrook, '58). Ephrussi and Slonimski ('50), and Slonimski ('53, '55) have also studied the anaerobically grown yeast cells with regard to their oxygen uptake, as well as the activities of several enzymes involved in their oxidative metabolism, and found in these cells very low oxidase and dehydrogenase activities, except for the malic, isocitric and alcohol dehydrogenases.

In the present work use was made of two methods that have not been previously applied to the question of oxidative metabolism and the corresponding enzymatic constitution of anaerobically grown yeast. One of the techniques was the use of ergosterol and oleic acid to make rapid and continuous anaerobic growth possible. The other method, by which quantitative data were obtained about the absorption intensities of the varying pigments present in these cells was the sensitive spectrophotometry developed by Chance (cf. review by Chance, '54). In this paper the absolute absorption spectra of anaerobically, as well as of aerobically grown cells, are presented together with the manometric data on these cells, representing the rates of oxygen consumption, and of CO_2 evolution in the presence and absence of oxygen. On the basis of these results, the oxidative enzymes and the Pasteur effect of the anaerobically grown yeast are discussed. Further papers in this series will deal with the enzymatic activities and the oxidized minus reduced difference spectra of this organism.

METHODS AND MATERIALS

Organism. Cells of *Saccharomyces cerevisiae,* strain LK2G12, a diploid strain, were used, a culture of which was originally received from Dr. S. Spiegelman, University of Illinois.

Media. The basic medium was adopted from the one used in the laboratory of Dr. S. Spiegelman and had the following composition:

40	gm	glucose (Difco Bacto-Dextrose)
5	gm	Difco Bacto-Peptone
2.5	gm	Difco Yeast Extract
6	gm	$(NH_4)_2SO_4$
2	gm	KH_2PO_4
0.34	gm	$CaCl_2 \cdot 2H_2O$
0.50	gm	$MgSO_4 \cdot 7H_2O$
6	ml	Na lactate, 60%
1000	ml	distilled water

This medium was used for aerobic cultures, while for anaerobic cultures it was supplemented the following way: (1) The glucose concentration was raised from 4% to 10%; and (2) 10 ml of an ergosterol and oleic acid stock solution was added to 1000 ml of the basic medium. The stock solution had the composition (after Andreasen and Stier, '54):

100 mg ergosterol
22 ml mono-oleate sulfate ester (Tween 80, Atlas Powder Co., Wilmington, Del.)
28 ml ethyl alcohol

This mixture was boiled until the ergosterol dissolved, then made up to 50 ml again with ethyl alcohol. The stock solution must be made up fresh at weekly intervals, as ergosterol seems to decompose quite rapidly in solution.

Another growth medium was also employed in a few experiments, following Stier, Scalf, and Peter ('50). This medium consisted of:

10%	glucose
0.5%	KH_2PO_4
7%	yeast extract
0.3%	wheat germ oil
0.03%	Tween 20

and will be referred to as the "high yeast extract — wheat germ oil medium."

In the latter medium the Tween 20 is added as an emulsifying agent, while in the former anaerobic medium Tween 80 serves as a source of oleic acid (Tween 20 is a mono-laurate, while Tween 80 is a mono-oleate sulfate ester, lauric acid being a saturated and oleic acid an unsaturated fatty acid).

Culture techniques. The aerobic cultures were set up by placing 70 ml medium in 250-ml Erlenmeyer flasks and shaking them on a rotary shaker at a rate of 160–180 cycles per minute at 26°C.

In the case of anaerobic cultures the main problem is the exclusion of traces of oxygen from the culture flask. The following procedure has proved satisfactory. In a one-liter suction flask 500 ml of the supplemented medium is placed, and the flask is closed at the top with a rubber stopper through which a glass tube is extended to the bottom of the flask for the gas inlet. The side outlet of the flask is connected with a rubber tube to a water trap consisting of a small stoppered bottle in which two glass tubes are inserted, one extending down to the bottom and the other a short way below the stopper. This whole assembly is autoclaved for 20 minutes at 15 lbs. pressure. After autoclaving high purity nitrogen gas is bubbled through it immediately. The purpose of placing the medium under nitrogen while still hot is to prevent oxygen from being dissolved in it. The nitrogen is passed through a cotton filter, and the connection of the nitrogen tank with the flask is through a short piece of rubber tubing that can be clamped off at the end of the gassing period. The medium is left to cool down while being bubbled with nitrogen, then the inoculum is introduced by taking the stopper out for a moment. Gassing is continued after this for 15 minutes. Then the rubber tubing at the gas inlet is clamped off, but the flask remains open for the evolution of CO_2 through the water trap. The medium is stirred during incubation (at 26°C) either with a magnetic stirrer or by placing the culture flask and the

water trap on a reciprocating shaker with a slow rate of shaking.

Inocula were obtained from two-day-old cultures in standing Erlenmeyer flasks containing 50 ml basic medium. These cultures were transferred every other day, and since they were open to the air but not shaken, the cells were of an intermediate type between aerobically and anaerobically grown ones. The inoculum size was 0.5 ml to 500 ml medium. It is important not to use too large an inoculum for the anaerobic cultures, since it is desirable to let the cells grow for at least 7 or 8 generations before harvesting in order to be sure of having cells with a true anaerobic enzyme composition.

Manometric techniques. Standard Warburg manometric procedures were followed as described by Umbreit, Burris and Stauffer ('49). CO_2 determinations were done following the direct method of Warburg. The Warburg vessels were of approximately 15 ml volume. The shaking rate was 130 cycles per minute with a 5 cm arc. The bath temperature was 26.4°C. For the measurements the vessels were set up with the following contents: 0.3 ml of cell suspension of a cell density of approximately 10^8 cells/ml (the cells were washed twice in distilled water and were suspended in M/15 KH_2PO_4 solution), and 0.2 ml of 20% glucose solution in the side arm. After all other reagents had been added, the final reaction volume in each vessel was made up to 3.0 ml with M/15 KH_2PO_4 solution (pH 4.5), so that the final cell density was 20–40 $\times$ 10^6 cells/ml and the final glucose concentration was 1.33% or 74 mM. The centerwell contained either 0.2 ml 30% KOH solution or the same volume of M/15 KH_2PO_4. For measuring the endogenous rates of O_2 uptake or CO_2 output, no glucose was added to the vessels and ten times as many cells were used. It usually took 40 minutes to set up an experiment, from the start of centrifugation of the yeast cultures to the tipping of the contents of the side arms. Before tipping 5 minutes temperature equilibration was allowed. The first reading was taken 10 minutes after tipping. The rate values were calculated from the readings between 10 and 40 minutes

after tipping, during which interval the rates stayed approximately constant. Cell counts were made at each experiment on the yeast suspension using a Spencer bright line haemacytometer. The results were expressed in units of μl gas/hr. × cell.

Spectroscopic techniques. In order to determine quantitatively the light absorption of intact yeast cells in the visible spectrum region, one must use either high sensitivity spectrophotometers (i.e., with photomultipliers) or spectrophotographic methods, because of the high scattering power of a cell suspension and the low concentration of the absorbing pigments. Both of these methods were used in the course of work. During the first part of the experiments a spectrophotographic method was worked out that consisted of placing 0.8-mm thick layers of frozen yeast paste in front of the slit of a Cornu-type spectrograph, and then obtaining optical density curves of the yeast cells by comparing the density of the photographed yeast spectra at each wavelength with a series of photographed spectra of known density neutral filters. This method was described in detail previously (Lindenmayer, '55), and was similar to that reported by Euler, Hellström, and Günther ('39), and by Chaix and Fromageot ('42).

In the latter part of this work it became possible to use the sensitive spectrophotometric equipment at the Johnson Research Foundation that offered great advantages over the previous method both in higher accuracy and speed of measurement. The results presented here were all obtained with this latter procedure. A split-beam sensitive spectrophotometer was used that was devised by Yang and Legallais on the basis of previous designs by Chance (Chance, '51; Yang and Legallais, '54; Yang, '54). The yeast suspensions for these measurements were prepared by washing the cells in distilled water, making up a cell suspension of 450 Klett density, then centrifuging 20 ml of this suspension and resuspending the cells in 2 ml buffer. This final suspension has ca. 10^8 cells/ml. To obtain the absorption spectra of the cells in the reduced state, one cuvette (1 cm diameter) of the split-beam spectro-

photometer contained 2.0 ml cell suspension reduced with a few crystals of solid $Na_2S_2O_4$, while in the other cuvette several layers of oiled filter paper were placed to balance the light scattering of the yeast cells. The absorption curves were corrected to a base line that connected the portions of the curve without absorption peaks. Spectra obtained this way are referred to as the absolute absorption spectra, as distinguished from the difference spectra where both cuvettes contain cells.

RESULTS

Growth data. Anaerobic growth was observed in three different media, and the cell yield was measured in the stationary phase of each culture. The unsupplemented basic medium was used to determine the yield of aerobic growth. The results are shown in table 1.

TABLE 1

Final cell densities under various culture conditions

ATMOSPHERE	MEDIUM	FINAL CELL DENSITY
		10^6 cells/ml
Aerobic	Basic	350
Anaerobic	Basic	4
Anaerobic	Basic supplemented with ergosterol and oleic acid	250
Anaerobic	High yeast extract — wheat germ oil medium	380

It is evident from these data that anaerobic growth was very limited in the basic medium. In this case the cell count was made 4 days after starting the culture, while in the other media the rapidly-growing phase was over and final cell densities were reached in less than 24 hours (the generation time for the aerobic culture was found to be 2.5 hrs. at 26°). The highest anaerobic yield was obtained in the 7% yeast extract — 0.3% wheat germ oil medium, in agreement with the findings of Andreasen and Stier ('56). Throughout this work the ergosterol-oleate supplemented basic medium was used for anaerobic cultures, rather than the high yeast extract — wheat germ oil medium, although the latter gave considerably higher yields. The reason for this is that including such

large amounts of yeast extract in the medium would raise the level of amino acids so high that they might also become a major source of carbon in competition with glucose. Such a shift in major nutrients would, in turn, influence the enzymatic composition of the cells to an extent that any comparison with the results of other studies, where conventional media were used, would be impossible. On the other hand, no defined micronutrient is yet known that would raise the anaerobic cell yield to the aerobic levels when added together with ergosterol and oleic acid.

Manometric data on aerobically grown cells. Measurements were performed at three growth stages of the aerobic cultures in the basic medium, the stages being characterized by the time elapsed from inoculation to the harvest of the cells, as well as by the cell densities. The first two measurements were within the exponential phase, and the third in the stationary phase. In each case, the rates of oxygen uptake were measured, as well as the rates of CO_2 output in air and in nitrogen atmosphere; furthermore, these rates were determined both in the presence and in the absence of glucose in the Warburg vessels. Thus both the "exogenous" and the "endogenous" metabolic rates were determined. The data obtained in the presence of exogenously supplied glucose were utilized for the calculation of the Meyerhof Quotients as well, furnishing information about the operation of the Pasteur effect in the cells. The following formula expresses a definition of the Meyerhof Quotient (Meyerhof, '21; Warburg, '26):

$$\text{M.Q.} = \frac{\text{fermentative cleavage products in } N_2 - \text{fermentative cleavage products in air}}{\text{oxygen consumption}}$$

The rate of fermentative CO_2 production will serve for our purposes as a measure for the appearance of fermentative cleavage products. The rate of fermentative CO_2 production in air ($Q^{air}_{CO_2\,ferm.}$) is obtained by subtracting the rate of oxygen uptake (Q_{O_2}) from the rate of total CO_2 production in air ($Q^{air}_{CO_2}$):

$$Q^{air}_{CO_2\,ferm.} = Q^{air}_{CO_2} - Q_{O_2}$$

assuming thereby that the Respiratory Quotient is unity under the conditions employed. Thus the Meyerhof Quotient is computed by the formula:

$$\text{M.Q.} = \frac{Q\ {}^{N_2}_{CO_2} - Q\ {}^{air}_{CO_2\,ferm.}}{Q_{O_2}}$$

The validity of using this quotient as an evidence for the operation of the Pasteur effect is discussed further below.

The results of these measurements are shown in table 2. The "exogenous" respiratory rate, i.e., the rate in the presence of exogenously supplied glucose, is more than doubled from the exponential to the stationary phase, while the exogenous fermentative rates, $Q\ {}^{air}_{CO_2\,ferm.}$ and $Q\ {}^{N_2}_{CO_2}$ decline with the age of the culture. This agrees with the findings of Slonimski ('53, p. 154) and of Ephrussi et al. ('56), who proposed explanations for this change in respiratory and fermentative activities during the development of a population.

The endogenous rate of respiration, on the other hand, shows a tenfold increase from the exponential to the stationary phase, which is very likely connected with the accumulation of endogenous reserves after rapid growth has ceased. The endogenous R.Q. remains near the value of one in cells at all stages, corresponding to the well-established fact that aerobically grown yeast cells are unable to ferment their endogenous carbohydrate reserves (Stier and Stannard, '36; Spiegelman and Nozawa, '45).

The values for the M.Q. were between 1.1 and 2.1. Since the criterion for the existence of the Pasteur effect in an organism is an M.Q. higher than ⅓, all three of the cell types can be considered as having normal Pasteur effect.

Absorption spectra of aerobically grown cells. Absolute absorption spectra were obtained with a sensitive recording spectrophotometer as described under the methods. The cells were in the early stationary phase, harvested after 24 hours of growth. The first spectrum (indicated by the solid line in figure 1) was taken of the washed cell suspension after glucose was added to it (to a concentration of ca. 0.01 M), and

TABLE 2

Respiratory and fermentative rates of cells grown aerobically in basic medium, at various stages of growth

TIME AFTER INOCULATION	NUMBER OF CELLS PER ML MEDIUM	WITH EXOGENOUS GLUCOSE					WITHOUT EXOGENOUS GLUCOSE			
		$Q_{O_2}^{air}$	$Q_{CO_2}^{air}$	$Q_{CO_2\ ferm.}^{air}$	$Q_{CO_2}^{N_2}$	M.Q.	$Q_{O_2}^{air}$	$Q_{CO_2}^{air}$	$Q_{CO_2\ ferm.}^{air}$	$Q_{CO_2}^{N_2}$
hrs.										
16.0	3.8×10^6	0.49	5.1	4.6	5.6	2.1	—	—	—	—
21.5	16.3×10^6	0.50	5.5	5.0	5.5	1.1	0.07	0.05	0	0
40.7	333×10^6	1.15	3.5	2.4	4.1	1.5	0.56	0.52	0	0

Q values in units of $10^{-6} \times \mu l$ gas/hr. $\times$ cell.

the cell suspension became anaerobic. The second spectrum was obtained after adding a few crystals of a reducing agent, sodium dithionite ($Na_2S_2O_4$), to the previous suspension (dashed line). The difference between the two spectra should be due to the pigments that can be reduced "chemically" but not "enzymatically," i.e., which are not reduced when the cells are without oxygen, but only in contact with a strong reducing agent that can enter the cells.

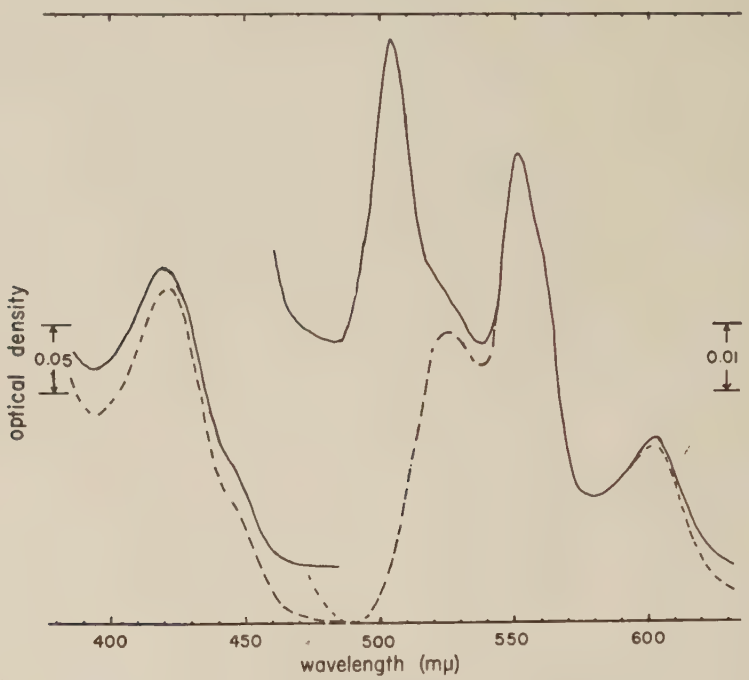

Fig. 1 The absolute absorption spectrum of *aerobically grown* yeast cells, stationary phase. Cell density: 10^8 cells/ml. Solid line: cells + glucose. Dashed line: cells + glucose + sodium dithionite. Optical density scale is different above and below 480 mμ, as indicated.

Both curves exhibit more or less pronounced absorption maxima at the following wave lengths: 420, 445, 525, 551 and 605 mμ, of which only the 551 mμ one is sharply defined. The curve taken in the presence of glucose alone shows in addition to these a large and sharp maximum at 503 mμ, as well as a

very broad shoulder between 450 and 550 mμ. All of the absorption maxima are easily identifiable in terms of the known cytochrome and flavin spectra (cf. Smith, '54), except the 503 mμ peak. Briefly, the 605 mμ peak is chiefly attributed to the α band of cytochrome *a;* the one at 560 mμ to the α band of cytochrome *b;* the one at 551 mμ to the overlapping α bands of cytochromes c_1 and *c;* the absorption around 525 mμ to the β bands of cytochromes *b,* c_1, and *c;* the 445 mμ peak chiefly to the γ band of cytochrome a_3; and the one at 420 mμ to the γ bands of cytochromes *b,* c_1, and *c.* Yeast cytochrome *c* peroxidase absorbs in the reduced state at 437 mμ, and in the oxidized state at 410 mμ (Abrams, Altschul and Hogness, '40), but these bands could not be identified here because of the absorption of the other pigments in the same region. The absorption in the broad interval between 450 and 550 mμ is due to the oxidized flavins present in the cells, which disappear upon the addition of a reducing agent.

The pigment that absorbs at 503 mμ has not been observed before, although many spectroscopic studies have been reported on baker's yeast. The main reason for this failure on the part of previous observers may have been the fact that this pigment cannot usually be found in starved commercial baker's yeast. On the other hand, we have observed it in fresh cultures of a number of various laboratory strains of baker's and brewer's yeasts. As will be seen further below, it is also present in the anaerobically grown cells. As figure 1 shows, this absorption band disappears in the presence of sodium dithionite, but no other absorption band has been found to appear paralleling this change.

Manometric data on anaerobically grown cells. Cells, grown in the ergosterol-oleate supplemented basic medium and harvested in the exponential or in the stationary phase, were used for the Warburg measurements, which were performed the same way as in the case of aerobically grown cells.

The results are presented in table 3. The rate of oxygen uptake in the presence of exogenously supplied glucose was in either phase less than 10% of the corresponding aerobically

TABLE 3

Respiratory and fermentative rates of cells grown anaerobically in supplemented basic medium, at various stages of growth

TIME AFTER INOCULATION	WITH EXOGENOUS GLUCOSE					WITHOUT EXOGENOUS GLUCOSE			
	$Q^{air}_{O_2}$	$Q^{air}_{CO_2}$	$Q^{air}_{CO_2\ ferm.}$	$Q^{N_2}_{CO_2}$	M.Q.	$Q^{air}_{O_2}$	$Q^{air}_{CO_2}$	$Q^{air}_{CO_2\ ferm.}$	$Q^{N_2}_{CO_2}$
hrs.									
16	0.05	10.4	10.35	10.6	5.0	0.06	0.12	0.06	0.04
28	0.09	6.0	5.9	6.2	3.3	0.11	0.19	0.08	0.12

Q values in units of $10^{-6} \times \mu$l gas/hr. $\times$ cell.

grown cells. The Q_{O_2} of exponential phase cells was again about half of that of the stationary phase ones. The exogenous fermentative rates, on the other hand, were about twice as high as those found in aerobically grown cells, but a decline can be seen from the exponential to the stationary phase, as in the aerobic cultures. The M.Q.'s, calculated on the basis of low respiratory rates, cannot be considered very accurate; it is still significant, however, that their values are higher (between 3 – 5) than are those observed for aerobically grown cells.

It is interesting to note that in these cells the rate of endogenous respiration is approximately the same as the exogenous rate. This fact was investigated in more detail, paying particular attention to some of the experimental difficulties. Using the manometric method for oxygen and CO_2 determinations, the difficulty arises when one is trying to establish accurately the very low rates of oxygen uptake in cells that are liberating at the same time large amounts of CO_2, which is the case with anaerobically grown yeast cells in the presence of exogenous glucose. The usual method of placing a paper wick in 20% KOH solution in the centerwell is not satisfactory in this instance; the liberated CO_2 is not absorbed rapidly enough, and therefore the manometric readings do not express the true rate of oxygen disappearance. This situation was corrected by raising the KOH concentration to 30%, and by placing 0.2 ml KOH solution and paper wicks in the two sidearms of the Warburg vessels in addition to the centerwell. In this way results were obtained that were essentially similar to those presented in table 3, and that agreed well, furthermore, with oxygen uptake measurements in the same kinds of cells by a polarized platinum electrode. Thus, it has been confirmed by various methods that the anaerobically grown cells had practically identical rates of oxygen uptake in the presence and absence of exogenous glucose, and this was found to be true even after the anaerobically grown cells have been aerated for a few hours in various media, but, of course, was not true for aerobically grown cells. It seems

that we are dealing here with cells in which the very weak respiratory pathway is saturated with the endogenously available substrates.

Another interesting aspect must be pointed out with regard to the endogenous rates shown in table 3, and that is the appearance of endogenous fermentation in these cells, both in air and nitrogen atmosphere. As mentioned above, endogenous fermentation has been reported by several workers to be entirely absent in baker's yeast, i.e., aerobically grown baker's yeast. Only Fales ('51) observed some endogenous fermentation in yeast cells during the first hour after removal from their growth medium, and attributed it to the presence of a fermentable substrate in the cells that is rapidly exhausted and is not identical with the substrate of respiration. Endogenous fermentation can also be induced in yeast cells by dinitrophenol (Rothstein and Berke, '52), and by azide (Brady and Duggan, '54), thus seemingly the uncoupling of phosphorylation makes endogenous fermentation possible without involving the necessity for a special substrate. Whatever the case may be, there is a significant difference in this regard between the aerobically and anaerobically grown cells, that could be exploited for further work on this subject.

Absorption spectra of anaerobically grown cells. The absorption curves, shown in figure 2, were obtained using early stationary phase cells grown anaerobically and harvested the same way as above. The dashed curve was taken in the presence of sodium dithionite, in addition to glucose, and shows a weak maximum at around 590 mμ, a well-defined one at 556 mμ, another weak one at about 530 mμ, and finally a large peak in the Soret region at 425 mμ and a shoulder around 445 mμ. The curve represented with the solid line was taken in the presence of glucose alone, and it shows two absorption bands in addition to the ones exhibited by the other curve. One is the large peak at 503 mμ, and the other a broad absorption region between 450 and 550 mμ.

The 590 and 556 mμ bands are identical with those that were found in brewer's yeast by Fink ('32), and designated

as the α bands of cytochrome a_1 and b_1 by Ephrussi and Slonimski ('50), and Chin ('50), while the 530 mμ maximum was attributed by them to the β band of cytochrome b_1. The absorption spectrum of the anaerobically grown yeast in the Soret region has not been investigated previously. The two

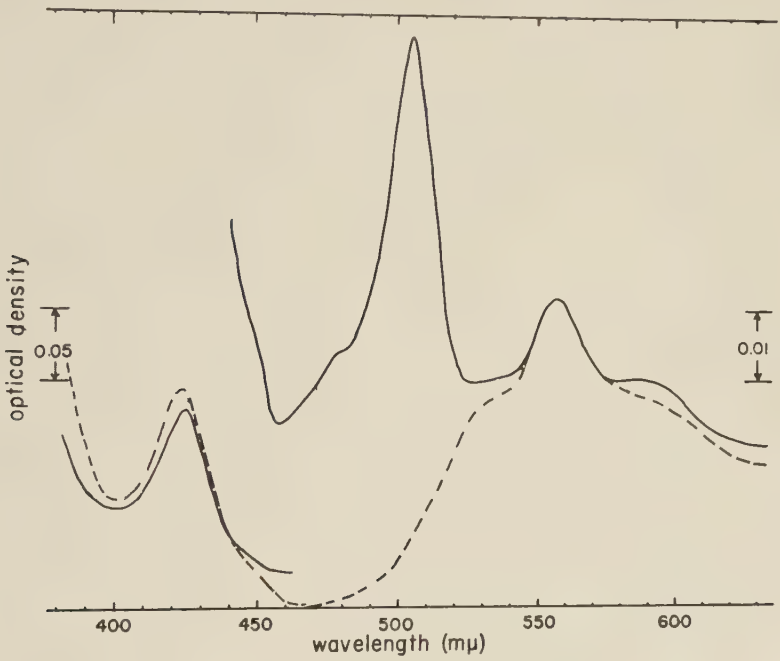

Fig. 2 The absolute absorption spectrum of *anaerobically grown* yeast cells, stationary phase. Cell density: 10^8 cells/ml. Solid line: cells + glucose. Dashed line: cells + glucose + sodium dithionite. Optical density scale different above and below 480 mμ, as indicated.

maxima in this region, at 445 and 425 mμ, can be regarded as the γ peaks of cytochromes a_1 and b_1, but this view requires further tests, particularly by a study of the behavior of these peaks in difference spectra (cf. following paper in this series).

The absorption bands that are missing in the cells reduced with sodium dithionite are the same two as those in the aerobically grown cells. The absorption between 450 and

550 mμ is again due to the oxidized flavins. It seems, however, that their contribution is considerably smaller than in the aerobically grown cells. The sharp band at 503 mμ has about the same intensity in anaerobically as in aerobically grown cells judging from the curves presented here, but this should by no means be considered to be the rule. The absorption intensity of this unidentified pigment varies greatly with the age of the culture and the subsequent treatment of the cells; it can entirely disappear under prolonged starvation, or its concentration can be built up much higher than that shown in the figures by aeration in glucose solution.

DISCUSSION

The study of the anaerobically grown yeast with respect to the oxidative processes and the enzymes involved is an interesting field not because of the role these processes play during growth but because of the potentialities these enzymes lend to the cells. In the course of anaerobic growth obviously no reaction or reaction sequence can take place that involves molecular oxygen. In these cells the presence of compounds that can combine with oxygen can be important, however, because it enables the cells to carry out oxidative metabolism as soon as oxygen becomes available, and also because these compounds can serve as the inducers or the precursors for the formation of the efficient oxidative systems that are found in the aerobically grown cells. Among the various oxidases of yeast, attention has been given by several investigators primarily to the origin of the cytochrome system, and thus the role of the cytochrome-like pigments in the anaerobically grown cells has become a subject of interest.

When exposed to oxygen, the cells raised under strict anaerobiosis exhibit immediately a low but measurable and reproducible rate of oxygen uptake. This rate, as the results indicate, is higher in cells harvested in the stationary phase than in the ones harvested in the exponential phase, and it is not affected by an exogenous supply of glucose. The ques-

tion is what are the enzymes that mediate the uptake of oxygen. It is possible that the low level of oxygen consumption of these cells, 10^{-7} µl O_2/hr. × cell, or in other terms 10 µl O_2/hr. × mg dry weight, is due to the activity of flavin enzymes or to some other oxidase and not to a heme enzyme. The fact, however, that several hemoproteins can be detected spectroscopically in these cells presents the possibility that they are the enzymes responsible for the utilization of oxygen.

The heme pigments that were provisionally named cytochromes a_1 and b_1 by Ephrussi and Slonimski ('50) and Chin ('50) should be considered first. The absorption maxima at 590 and 556 mµ were attributed to the α bands of these pigments, the maximum at 530 mµ to the β bands, while the 445 and 425 mµ peaks shown in figure 2 can now be attributed to their γ bands (the 425 mµ peak includes very probably the absorption of another pigment as well). These bands are present when the cells are in the reduced state and, as will be shown in a following paper, they disappear when the cells are vigorously aerated. The oxidation and reduction of the cytochromes a_1 and b_1 are thus closely connected with the presence or absence of oxygen in the cells. This means either that one or both of them can directly combine with oxygen, or that they are oxidized and reduced by another enzyme that combines with oxygen. The first seems to be the more likely one in view of the evidence, presented in the following paper, that no cytochrome a_3 can be detected in these cells.

The absorption maximum at 503 mµ presents a special problem. It is found both in aerobically and in anaerobically grown cells of the yeast strain used in this work. The concentration, i.e., the intensity of the absorption peak at 503 mµ, is increased considerably when the cells are aerated in the presence of glucose, while it decreases upon prolonged starvation. This peak does not change rapidly with the presence or absence of oxygen in the cell suspension, but it disappears instantaneously from the absorption spectrum when sodium dithionite or potassium cyanide is added to the cells. The

chemical nature of this pigment has not yet been established. It certainly does not belong either to the cytochrome-like or to the flavin-like compounds, and it does not participate directly in the oxidative processes as those pigments do. Further data concerning this pigment will be presented with the difference spectra.

The Pasteur effect, i.e., the suppression of fermentation by respiration, should also be considered in connection with the oxidative metabolism of this organism. As mentioned above, we are using here the Meyerhof Quotient as a criterion of the Pasteur effect, calculated from the "exogenous" rates of the fermentative CO_2 production, in the presence and absence of oxygen, and of the rate of oxygen uptake. This means that we are determining the Pasteur effect by comparing the appearance of the fermentative cleavage products, rather than by the uptake of carbohydrates, between the two conditions. The former procedure has been advocated by Burk ('39), but not by Dixon ('37). Three other assumptions are implicit with regard to the Meyerhof Quotient, these being: (1) that respiration in these cells proceeds with a Respiratory Quotient of unity; (2) that the rates of endogenous respiration and fermentation are negligible in the presence of exogenously supplied glucose; and (3) that equivalent amounts of CO_2 and ethyl alcohol are produced in fermentation. These assumptions are not necessarily fulfilled in all the various cell types studied and under all conditions, but they are in general agreement with the results of numerous studies of yeast metabolism.[3]

The point that becomes evident from the data presented here is that anaerobically grown cells show consistently higher values for the Meyerhof Quotient than the aerobically grown ones do. The data given in table 3, being representative of a large number of experiments, indicate an average Meyerhof Quotient of around 4 for the anaerobically grown cells.

[3] Pasteur effect, as the term is employed here, refers only to the *reversible* effect of respiration on fermentation, and not to changes over a long period of time, which may be due to changes in the enzymatic composition of the cells.

The determination of this quotient in this case is subject to a number of difficulties and errors. The cells have a very low respiratory and a high fermentative rate, and the rate of aerobic fermentation is only 2–5% lower than the anaerobic rate. The problem of measuring the small amounts of oxygen taken up during the same period when large quantities of CO_2 are liberated by the cells, and the answer to it, have been described above. Another source of error could have been the fact that the Warburg vessels used for Q_{O_2} determination did not contain any CO_2, while the other vessels in the same experiment that are set up without KOH solution do have CO_2. If the yeast cells can fix or "recycle" CO_2, the metabolic situation would not be comparable in the two vessels. Therefore, some CO_2 determinations were carried out in the course of this work by Warburg's indirect, or two-vessel method (Umbreit, Burris and Stauffer, '49). The indirect and the direct methods of Warburg gave identical results. Thus the measurements giving high values for the Meyerhof Quotient in the anaerobically grown cells seem to be valid. Previous workers have reported similar results with yeast strains deficient in cytochromes. Stier and Castor ('41) have reported high Meyerhof Quotients in their cyanide induced yeast mutants, while Meyerhof and Davidson ('52) and Slonimski ('53, p. 186) obtained Meyerhof Quotients of 8–15 and around 4, respectively, working with a "petite" strain of yeast.

The observation is, therefore, that yeast cells devoid of the cytochrome system exhibit Meyerhof Quotients of 4 or higher, while the normal, aerobically grown cells give values of about two or lower. If the Pasteur effect, i.e., the slowing down of glycolysis, is brought about by a localized lack of phosphate acceptors when respiration is operating, as it was proposed first by Johnson ('41) and Lynen ('41), then the value of the Meyerhof Quotient will be related to the rates at which phosphorylation proceeds in the various enzyme systems of the cell, and to the rates at which the phosphorylated compounds diffuse from one system to the other, or are used up. When yeast cultures are compared that have entirely different

respiratory systems, i.e., the cytochrome system in the case of aerobically grown cells and an unidentified system in the case of the anaerobically grown cells and the mutant strains, the difference in the phosphorylation and the diffusion rates evidently becomes expressed by the difference in the Meyerhof Quotients.

SUMMARY

Cells of *Saccharomyces cerevisiae,* strain LK2G12, were grown anaerobically in the presence of ergosterol and oleate. Their overall metabolism was studied by measuring manometrically the rates of oxygen consumption, and of carbon dioxide evolution in air and in nitrogen atmosphere. The absorption spectra of the intact cells were obtained with a recording spectrophotometer. These results were compared with corresponding ones for aerobically grown cells. The presence of cytochromes a_1 and b_1 is related to the oxygen uptake and to the Pasteur effect of the anaerobically grown cells. A new pigment, with absorption maximum at 503 mμ, is observed, which is present in the intact cells when aerated in glucose solution, and disappears when dithionite or cyanide is added.

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LITERATURE CITED

ABRAMS, R., A. M. ALTSCHUL AND T. R. HOGNESS 1940 Cytochrome *c* peroxidase. J. Biol. Chem., *136:* 777–794.

ANDREASEN, A. A., AND T. J. B. STIER 1953 Anaerobic nutrition of *Saccharomyces cerevisiae.* I. Ergosterol requirement for growth in a defined medium. J. Cell. and Comp. Physiol., *41:* 23–36.

ANDREASEN, A. A., AND T. J. B. STIER 1954 Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium. Ibid., *43:* 271–281.

———— 1956 Anaerobic nutrition of *Saccharomyces cerevisiae*. III. An unidentified growth promoting factor and its relationship to the essential lipid requirements. Ibid., *48:* 317–328.

BOREI, H., AND A. SJÖDÉN 1943 Variations in the cytochrome *c* content of top yeast with cultural conditions. Arkiv Kemi, Mineral. Geol. *16A* (19): 1–17.

BRADY, T. G., AND P. F. DUGGAN 1954 Initiation of endogenous fermentation in baker's yeast by azide in high concentration. Biochem. J., *58:* XXII.

BROWN, A. J. 1905 The influences regulating the reproductive functions of *Saccharomyces cerevisiae*. J. Chem. Soc. (London), *87:* 1395.

BURK, D. 1939 A colloquial consideration of the Pasteur and neo-Pasteur effects. Cold Spring Harbor Symp. Quant. Biol., *7:* 420–459.

CHAIX, P., AND C. FROMAGEOT 1942 Spectrographie quantitative de microorganismes. Bull. Soc. Chim. Biol., *24:* 1259–1268.

CHANCE, B. 1951 Rapid and sensitive spectrophotometry. Rev. Sci. Instrum. *22:* 619–638.

———— 1954 Spectrophotometry of intracellular respiratory pigments. Science, *120:* 767–775.

CHIN, C. H. 1950 Effect of aeration on the cytochrome systems of the resting cells of brewers' yeast. Nature, *165:* 926–927.

DIXON, K. C. 1937 The Pasteur effect and its mechanism. Biol. Rev. Cambridge Philos. Soc., *12:* 431–460.

EPHRUSSI, B., AND P. P. SLONIMSKI 1950 La synthèse adaptive des cytochromes chez la levure de boulangerie. Biochim. Biophys. Acta, *6:* 256–267.

EPHRUSSI, B., P. P. SLONIMSKI, Y. YOTSUYANAGI AND J. TAVLITZKY 1956 Variations physiologiques et cytologiques de la levure au cours du cycle de la croissance aerobie. C. R. Lab. Carlsberg, Sér. Physiol., *46:* 87–102.

EULER, H. VON, H. FINK AND H. HELLSTRÖM 1927 Über das Cytochrom in Hefezellen. II. Hoppe-Seylers Z. physiol. Chem., *169:* 10–51.

EULER, H. VON, H. HELLSTRÖM AND G. GÜNTHER 1939 Oxydations-Systeme in Oberund Unter-Hefe. Ibid., *258:* 47–56.

FALES, F. W. 1951 The assimilation and degradation of carbohydrates by yeast cells. J. Biol. Chem., *193:* 113–124.

FINK, H. 1932 Klassifizierung von Kulturhefen mit Hilfe des Cytochromspektrums. Hoppe-Seylers Z. physiol. Chem., *210:* 197–219.

FINK, H., AND E. BERWALD 1933 Über die Umwandlung des Cytochromspektrums in Bierhefen. Biochem. Z., *258:* 141–146.

JOHNSON, M. J. 1941 The role of aerobic phosphorylation in the Pasteur effect. Science, *94:* 200–202.

LINDENMAYER, A. 1955 Adaptive aspects of glucose metabolism in baker's yeast. Ph. D. Thesis, Univ. of Michigan, Ann Arbor.

LINDENMAYER, A., AND R. W. ESTABROOK 1958 Low temperature spectral studies on the biosynthesis of cytochromes in baker's yeast. Arch. Biochem. Biophys., *78:* 66–82.

LYNEN, F. 1941 Über den aeroben Phosphatbedarf der Hefe. I. Ein Beitrag zur Kenntnis der Pasteur'schen Reaktion. Liebigs Ann. Chem., *546:* 120–141.

MEYERHOF, O. 1921 Die Energieumwandlungen im Muskel. IV. Über die Milchsäurebildung in der zerschnittenen Muskulatur. Pflügers Arch. gesamt. Physiol., *188:* 114–160.

MEYERHOF, O. 1925 Über den Einfluss des Sauerstoffs auf die alkoholische Gärung der Hefe. Biochem. Z., *162:* 43–86.

MEYERHOF, O., AND H. M. DAVIDSON 1952 Pasteur effect in yeast lacking cytochrome oxidase. Fed. Proc., *11:* 258.

PASTEUR, L. 1876 Études sur la bier. Paris: Gauthier-Villars.

ROTHSTEIN, A., AND H. BERKE 1952 Endogenous alcoholic fermentation in yeast induced by 2,4-dinitrophenol. Arch. Biochem. Biophys., *36:* 195–201.

SLONIMSKI, P. P. 1953 La formation des enzymes respiratoires chez la levure. Actualites Biochimiques No. 17, Masson et Cie., Paris.

———— 1955 Adaptation respiratoire: développement du système hémoprotéique induit par l'oxygene. Proc. Third Internatl. Congr. Biochem., (1956). Academic Press, New York, pp. 242–252.

SMITH, L. 1954 Bacterial cytochromes. Bacteriol. Rev., *18:* 106–130.

SPIEGELMAN, S., AND M. NOZAWA 1945 On the inability of intact yeast cells to ferment their carbohydrate reserves. Arch. Biochem., *6:* 303–322.

STIER, T. J. B., AND J. G. B. CASTOR 1941 On the production and use of permanently altered strains of yeast for studies of *in vivo* metabolic organization. J. Gen. Physiol., *25:* 229–233.

STIER, T. J. B., R. E. SCALF AND M. C. BROCKMAN 1950 An all-glass apparatus for the continuous cultivation of yeast under anaerobic conditions. J. Bacteriol., *59:* 45–49.

STIER, T. J. B., R. E. SCALF AND C. J. PETER 1950 Edible oils as sources of lipid anaerobic growth factors for distiller's yeast. J. Cell. and Comp. Physiol., *36:* 159–165.

STIER, T. J. B., AND J. N. STANNARD 1936 The metabolic systems involved in dissimilation of carbohydrate reserves in baker's yeast. J. Gen. Physiol., *19:* 479–494.

TRAUTWEIN, K., AND J. WASSERMANN 1930 Über die Atmung und Gärung von ober- und untergärigen Bierhefen. Biochem. Z., *229:* 128–153.

UMBREIT, W. W., R. H. BURRIS AND J. F. STAUFFER 1949 Manometric Techniques and Tissue Metabolism. Burgess Publ. Co., Minneapolis.

WARBURG, O. 1926 Über die Wirkung von Blausäureäthylester (Äthylcarbylamin) auf die Pasteur'sche Reaktion. Biochem. Z., *172:* 432–441.

———— 1927 Über den Stoffwechsel der Hefe. Ibid., *189:* 350–353.

WHITE, J., AND D. J. MUNNS 1951 The effect of aeration and other factors on yeast growth and fermentation. Wallerstein Lab. Commun., *14:* 199.

WINDISCH, F. 1932 Die Bedeutung des Sauerstoffs für die Hefe und ihre biochemische Wirkungen. Biochem. Z., *246:* 332–382.

YANG, C.-C. 1954 A rapid and sensitive recording spectrophotometer for the visible and ultraviolet region. II. Electronic circuits. Rev. Sci. Instrum., *25:* 807–813.

YANG, C. -C., AND V. LEGALLAIS 1954 A rapid and sensitive recording spectrophotometer for the visible and ultraviolet region. I. Description and performance. Ibid., *25:* 801–807.

THE AMINO ACID COMPOSITION IN RELATION TO CELL GROWTH AND CELL DIVISION IN SYNCHRONIZED CULTURES OF TETRAHYMENA PYRIFORMIS [1]

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FIVE FIGURES

INTRODUCTION

Detailed studies on the amino acid composition and nitrogen metabolism of the ciliated protozoan, *Tetrahymena pyriformis*, strain E, have recently been reported by Wu and Hogg ('52), who demonstrated that this organism has a distinctive amino acid pattern comparable to other animals and micro-organisms. This pattern, however, varies for some of the amino acids if the cultures are maintained in different media, such as proteose peptone or a synthetic medium. In addition to these reports on the composition of the total cellular amino acids, Wu and Hogg ('56) have also analyzed the free and nonprotein amino acids and have found a fairly high level of free amino acids and an amino nitrogen content which is almost one fifth of the total nitrogen content of the cell.

All these studies by Wu and Hogg were carried out on cultures harvested after 4 to 5 days of growth, when the cells were in the maximum stationary phase. In this stage of population growth there is practically no cell multiplication occurring, and the growth rate is almost zero (Ormsbee, '42, Scherbaum, '56). It is not known, however, whether this state of almost complete lack of cytoplasmic synthesis and cell division is reflected in the amino acid pattern of the cell protein or in

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the composition and pool size of the free amino acids. Therefore, the result of these analyses should be compared with samples harvested during exponential multiplication. In the exponential growth phase the cell mass doubles every 170 minutes, and 7 to 10% of the cell population is always in the visible stage of fission (Scherbaum, '57a,b). Such a comparison should allow us to distinguish between the amino acid content of the two systems, in which cell growth and cell division are present in one and absent in the other.

The above mentioned comparison, valuable as it might be for determining differences between dividing and non-dividing cells, would not give us direct information concerning any differences which might exist during the reproductive cycle of individual cells. However, this can be accomplished by a recently developed method for induction of synchronous cell division in a mass culture. By these means, processes pertaining to increase of mass can be separated from those which are connected with the process of division (Scherbaum and Zeuthen, '54, '55; Holz, Scherbaum and Williams, '57). In this connection it is of interest to note that in the early development of the sea urchin, *Paracentrotus lividus* in which mass does not increase, changes of the amino acid concentration are related directly to cell division (Kavanau, '54).

The present investigation was undertaken to obtain information on the pattern of the free and protein amino acids at various characteristic stages of population growth, namely, (1) the logarithmic phase characterized by rapid synthesis and multiplication; (2) the maximum stationary phase in which there is no increase in cell mass and no multiplication; (3) at various stages of induced synchronous division.

Mass cultures were synchronized by the method of Scherbaum and Zeuthen ('54, '55). The cultures are subjected to a series of heat shocks for 7 hours. During the first half of the treatment the average cell volume at first increases at the normal rate. However, this growth in cell volume is increasingly inhibited in the latter part of the treatment. After the end of the last temperature shock a short characteristic lag

period occurs, followed by a burst of synchronous cell division. Such cultures were sampled at various stages during and after the temperature treatment to find out whether (1) the decreased rate of synthesis in cell mass during treatment might be reflected in a change of the amino acid pattern of the cells, and whether (2) the cytological changes occurring prior and during cell division are linked to detectable changes in the concentration of the free amino acid pool.

METHODS

A. Organism and growth conditions

Tetrahymena pyriformis GL was grown in a peptone medium of the following composition: 2% (w/v) proteose peptone (Difco), 0.1% (w/v) liver fraction L (Wilson Laboratories), 0.5% (w/v) bacto-dextrose (Difco). Sulfates and chlorides were added as in basal medium A of Kidder and Dewey ('51). Stock cultures were maintained in 3 ml of medium in upright test tubes in the dark. Test tube cultures three to 4 days old were used as inocula (1 ml) of 100 ml of medium in 250 ml Erlenmeyer flasks. Seventy ml of such cultures (two days old) were transferred asceptically into 7 liters of medium and grown at 28°C for approximately 12 hours in a flat rectangular stainless steel container, 80 × 42 × 12 cm in size, which served as a culture tank, with a lid of pyrex glass plate. Prior to inoculation the whole unit was sterilized by autoclaving at 15 pounds for 15 minutes. Two holes in the lid were used for inoculation and aeration with sterile air. A plastic housing built around the tank facilitated aseptic inoculation of the experimental culture. The container was gently tilted around its minor axis at 18 strokes per minute to ensure proper aeration of the 21 mm layer of the culture medium. A stainless steel tube (8 mm in diameter, approximately 7 m long) was bent and built in, so that it more or less covered the bottom of the container. This tube was connected to a system of valves, an interval timer, two thermostatically controlled water baths and a pumping system. By this means

water from either bath could be pumped through the stainless steel tube in the bottom of the culture tank, thereby changing the temperature to the optimum for growth (29°C) or the level of heat shocks (34°C). A complete change of temperature required 6 to 10 minutes. By means of a tap near the bottom of the container, samples of 5 ml were removed from the experimental culture at regular intervals for direct cell counts (Scherbaum, 57c).

B. Harvesting of cells and preparation of dry powder

For each stage of population growth under investigation, samples of 0.5 to 2 liters of culture fluid were removed and centrifuged in 600-ml glass tubes at 280 × g for 5 to 7 minutes at room temperature. The supernatant fluid was poured off and the cells washed once in tap water (dilution approximately 1:100), centrifuged, and collected from the specially designed elongated funnel-shaped bottom of the tube. The concentrated cell suspension was pipetted into 15-ml conical glass tubes and centrifuged at 250 × g for 5 minutes. The supernatant and debris were removed by suction, and the residual densely packed cells were heated in a water bath at 95°C for 5 minutes. The cells were then transferred to petri dishes and dried at 60°C. After pulverization, the preparations were stored in a desiccator. Some of the samples were ashed by ignition at 500°C in a furnace for 12 hours and the ash content was determined. It was 3.9 to 4.3% of the dry weight of the preparation.

C. Isolation of free amino acids

The method followed (to isolate free amino acids) was the procedure given by Awapara ('48). Dry powder (100 mg) was homogenized in a ground glass homogenizer with 2 ml of hot, glass distilled water. After 20 minutes at 95°C, 8 ml of absolute alcohol was added and the material was centrifuged. The residue was washed with 4 ml of 80% alcohol. Both supernatants were pooled and transferred to a separatory funnel; 34 ml of chloroform was added and thoroughly shaken. The

clear aqueous supernatant phase which separated from the ethanol-chloroform phase on standing was removed, evaporated and either (1) dissolved in 0.1 ml of 10% isopropanol for chromatography or (2) hydrolyzed in HCl as described for the protein fraction below. Aliquots consisting of from 4 to 8 mg dry weight of cells were used for each chromatogram.

D. Isolation of amino acids from peptides and proteins

The alcohol precipitate in the described procedure was placed in 20 ml of 10% sodium chloride and kept at 85°C for 6 hours to extract the nucleic acids. The samples were filtered, and each residue was washed thoroughly with distilled water and hydrolyzed in 20 ml 3 N HCl for 7 hours at 15 pounds pressure. The hydrolysates were filtered, concentrated to dryness on a steam bath and redissolved in 10% isopropanol. Amounts consisting of from 1 to 2 mg dry powder were applied on each chromatogram.

E. Paper chromatography

Whatman no. 1 filter paper, size $18\frac{1}{4} \times 22\frac{1}{2}$ inches, was used for two-dimensional chromatography. The paper was washed in a plexiglass tray in 2 N acetic acid for two days and rinsed with distilled water for 4 days. The paper was developed first in the short dimension in the following solvent: butanol (4v)–acetic acid (1v)–water (1v). In the second dimension, phenol (4v)–water (1v) was used, and petri dishes containing 0.5% ammonia were placed on the bottom of the chromatographic chamber. In order to retard decomposition of the phenol 0.04% 8-hydroxyquinoline was added (Wellington, '51). For both solvents the exposure time was approximately 15 hours at 29 ± 1°C. To locate the spots the chromatograms were briefly washed in acetone–ether (v/v) to remove traces of phenol and dipped into a solution of 0.25% (w/v) ninhydrin in acetone (Toennies and Kolb, '51). The color was developed by heating the chromatograms at 35°C in presence of water vapor for one hour. Contact prints of the chromato-

grams were made on kodagraph paper by illumination with yellow light (Kodak Series A). Photographic reproductions of these prints are presented in this paper.

F. Identification of the amino acids

From known standards the Rf values were calculated, and as a basis for comparison C-14 labelled amino acids were run, concommitant with the experimental samples. Color reactions were carried out according to Lyttle and Moffat ('57).

G. Quantitative estimation of the amino acids

Developed and washed chromatograms were dipped into 0.01% ninhydrin in acetone and dried for 10 minutes at 65°C. The spots and three blanks were cut out on each sheet. Each paper spot was flushed with 250 μl borate buffer in methanol, dried for 5 minutes at 40°C (Connell et al., '55), rolled with help of forceps, and transferred to test tubes. For the color reaction, a combination of ninhydrin, hydrindantin, and methylcellosolve in an acetate-sodiumacetate buffer was used (Connell et al., '55). This reagent was prepared fresh the day before use. The development of the color was carried out in 2 ml of the reagent by boiling for 20 minutes. The extracted paper was washed in 50% alcohol, and the samples made up to a known volume. The color intensities were measured in a Beckman spectrophotometer at 570 mμ (proline at 440 mμ). The extinction coefficients given by Connell et al. ('55) were used for the calculation of the molar concentration of the amino acids.

EXPERIMENTS AND RESULTS

A. Characterization of the growth steps chosen

The 7-liter mass culture was grown for approximately 12 hours at constant optimum temperature for multiplication at 28–29°C as described above (see methods). The first sample was taken when the population density was 30–80 $\times$ 10^3 cells

per ml (termed growth stage 1 hereafter). This sample served as a control of normal exponential multiplication with an average cell volume of about 26,000 μ^3 (Scherbaum, '56) and a division index of 5 to 8%. Immediately after removal of the first sample the heat treatment was started. In this procedure the temperature of the culture medium was changed in a rhythmic fashion: each cycle consisting of one half hour at 33.9°C and one half hour at 28.5°C. After the second heat shock the division index drops practically to zero, but the cells continue to grow. At the end of the seventh shock, that is 6.5 hours after the treatment started, the next sample was taken (growth stage 2). At this stage the individual cells have increased in size more than three times and their dry weight has at least doubled (Scherbaum, '56, '57). The next sample (growth stage 3) was taken from the culture one hour after the end of the last temperature shock during the induced lag, 20 minutes prior to the synchronous division. This recovery period is temperature-dependent in a characteristic fashion, and kinetic studies have suggested that (the same) processes might occur which are essential in preparing the normal cell for division (Scherbaum, 57a; Scherbaum and Zeuthen, '53). Forty-eight hours after inoculation the last sample (growth stage 4) was removed from the incubator. In this phase the population density is close to one million cells per milliliter and practically no dividing cells are found in the culture. The average cell volume is reduced (i.e., to 19,000 μ^3) when compared with cells in the early logarithmic phase of growth (Scherbaum, '56).

B. Concentration of peptide and protein amino acids

The chromatogram presented in figure 1 shows the typical amino acid pattern of the hydrolyzed ethanol-precipitable peptides and proteins of a heat-treated culture prior to the synchronous division (growth step 3). Fifteen spots were located and 10 amino acids were separated and identified. In order of increasing Rf values in phenol these are: aspartic acid,

cystine, glutamic acid, serine, glycine, threonine, alanine, tyrosine, arginine and proline. On three spots two or more amino acids were found. These are: lysine and histidine; valine and methionine; leucine, isoleucine and phenylalanine. This pattern of the composition of the peptide and protein hydrolysate seems to be retained on a qualitative basis in all of the growth stages studied; that is, no new spot was found in any of the 4 stages characterized above.

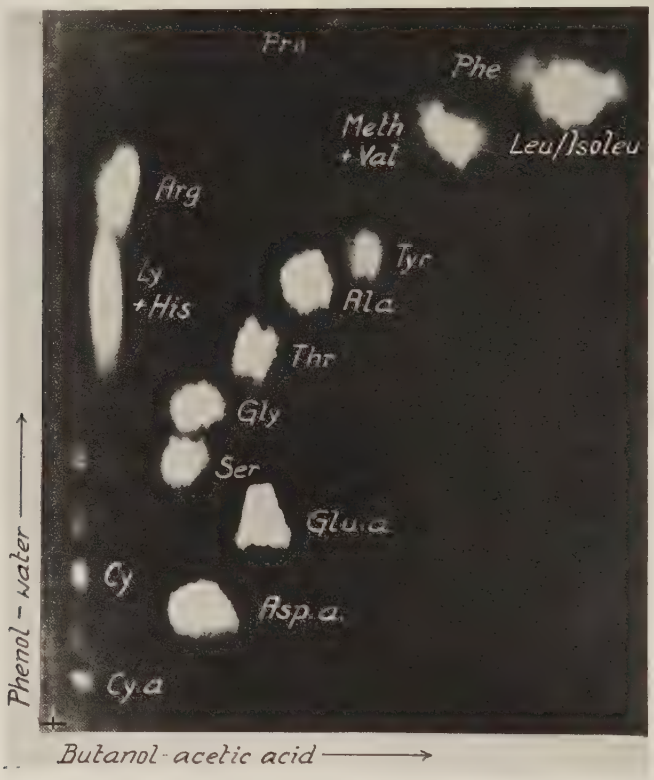

Fig. 1 Paper chromatogram of a protein hydrolysate of a sample prior to synchronous division corresponding to 1 mg dry weight. Development in butanol–acetic acid–water in the first dimension and phenol–water in the second dimension. The following abbreviations are used: Ala = alanine, Arg = arginine, Asp. a. = aspartic acid, V = cysteic acid, Cy = cystine, Glu.a. = glutamic acid, Gly = glycine, His = histidine, Leu/Isoleu = leucine/isoleucine, Ly = lysine, Meth = methionine, Phe = phenylalanine, Pro = proline, Ser = serine, Thr = threonine, Tyr = tyrosine, Val = valine. The cross in the lower left corner is the starting point.

A quantitative estimation of the concentration of the isolated amino acids was carried out according to the method described by Connell et al. ('55). The results are shown in figure 2. The optical densities per milligram dry weight of cells are plotted for each isolated amino acid or group of amino acids at the various growth stages. The average values for two parallel experiments are presented. A correction for the loss during the chromatographic and isolation procedure (8–15%) is included. The dotted lower part of the histograms gives the smallest amounts found while the different symbols above represent the 4 growth steps chosen. The standard deviation for the amounts between these stages is 6.15%. It appears that no significant change of the normal amino acid pattern occurs under the various growth conditions.

The micromolar concentrations of these amino acids were calculated with help of the micromolar extinction coefficients given by Connell et al. ('55). The extinction coefficients for the amino acids which could not be separated are almost identical; therefore, the error introduced in this calculation seems negligible. The results are shown in table 1. Aspartic acid, glutamic acid, and the combined leucine/isoleucine/phenylalanine are most abundant and represent almost 50% of the total sample.

C. Concentration of the free amino acids

One aliquot of the fraction containing all the amino acids and smaller peptides which are soluble in 80% ethanol was subjected to paper chromatographic analysis. Another aliquot was hydrolyzed as outlined for the protein residue. The results are shown in figures 3a, b. Figure 3a shows the contact print of (a chromatograph of) an unhydrolyzed sample of growth stage 3 (prior to division). Eighteen spots could be located, while upon hydrolysis only 16 spots were found. Glutamine, asparagine and an unidentified compound X-1 could not be recovered after acid hydrolysis (fig. 3b). On the other hand this hydrolysis of the ethanol extracts caused a marked

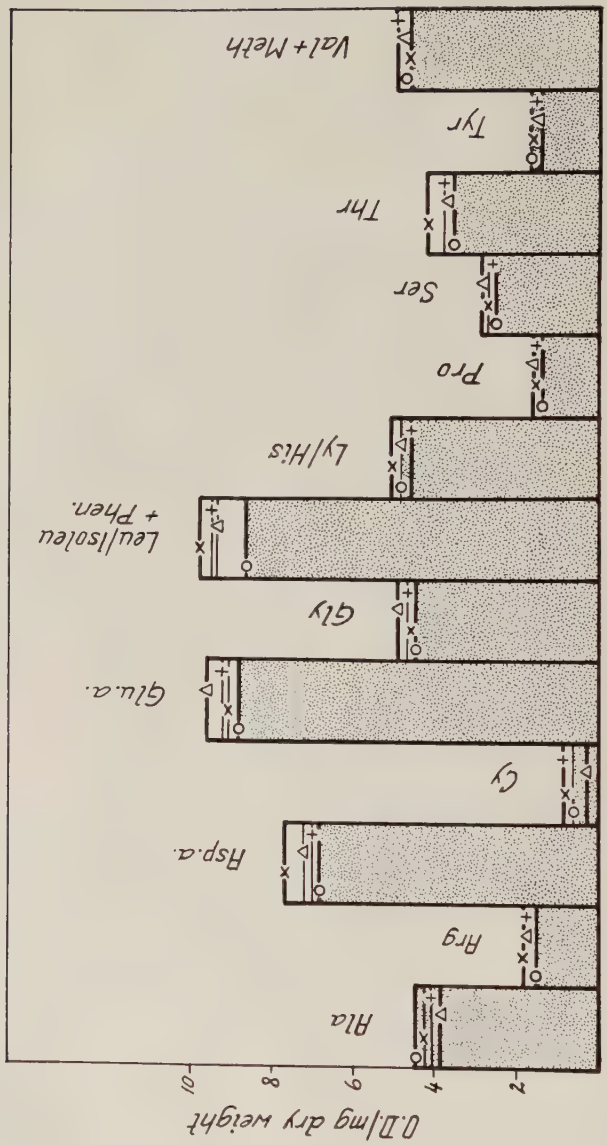

Fig. 2 Histograms of isolated amino acids from the protein fraction. The values on the ordinate (O.D) give the total absorption, measured at 570 mμ (440 mμ for proline) for the amino acids listed. The symbols refer to the following growth stages: 1, exponential multiplication (—O—); 2, after seven heat shocks (— X —); 3, one hour after end of treatment, prior to the synchronous division, (— Δ —); 4, approximately 18 hours later in the maximum stationary phase, (— + —). The shaded areas are values found for exponential growth.

increase in strength of the spots given by aspartic acid, glutamic acid, glycine and threonine. Comparison of X-1 with various standards showed the same Rf values as for oxidized glutathione. The peptide nature of this compound is further shown in its disappearance in the hydrolysate (fig. 3b).

TABLE 1

Amino acid composition of protein hydrolysates at various growth stages

1, exponential multiplication; 2, after end of heat treatment; 3, prior to synchronous division, 4, in the maximum stationary phase. Values are given in micromoles per gram dry weight.

AMINO ACID	GROWTH STAGE			
	1	2	3	4
	(μM/g)			
Alanine	205.9	196.7	179.7	187.0
Arginine	70.5	89.2	85.9	81.7
Aspartic acid	339.6	381.1	356.4	346.5
Cystine	32.5	40.0	17.5	45.0
Glutamic acid	402.2	415.0	440.4	419.0
Glycine	213.2	216.1	234.5	215.6
Leucine/Isoleucine/Phenylalanine	415.3	471.2	447.8	466.5
Lysine/Histidine	182.5	214.1	206.2	194.5
Proline	85.5	77.0	80.0	81.0
Serine	129.2	137.6	143.0	136.1
Threonine	175.1	202.3	176.5	183.7
Tyrosine	93.7	86.4	82.2	83.8
Valine/Methionine	229.1	222.0	225.8	236.1

A quantitative estimation of the concentration of the amino acids in the unhydrolyzed sample is given in table 2 and in figure 4. The average values of two experiments were calculated. The concentrations of the amino acids vary more between the experiments and from one growth stage to the other than the values found for the protein amino acids (SD = 16.9%). This is also evident from the same data calculated on a concentration basis and given in table 2. These variations cannot be attributed to the experimental error alone. Aside from these variations no systematic change in the concentrations of the amino acids analyzed could be attributed to the observed changes occuring at the cellular level.

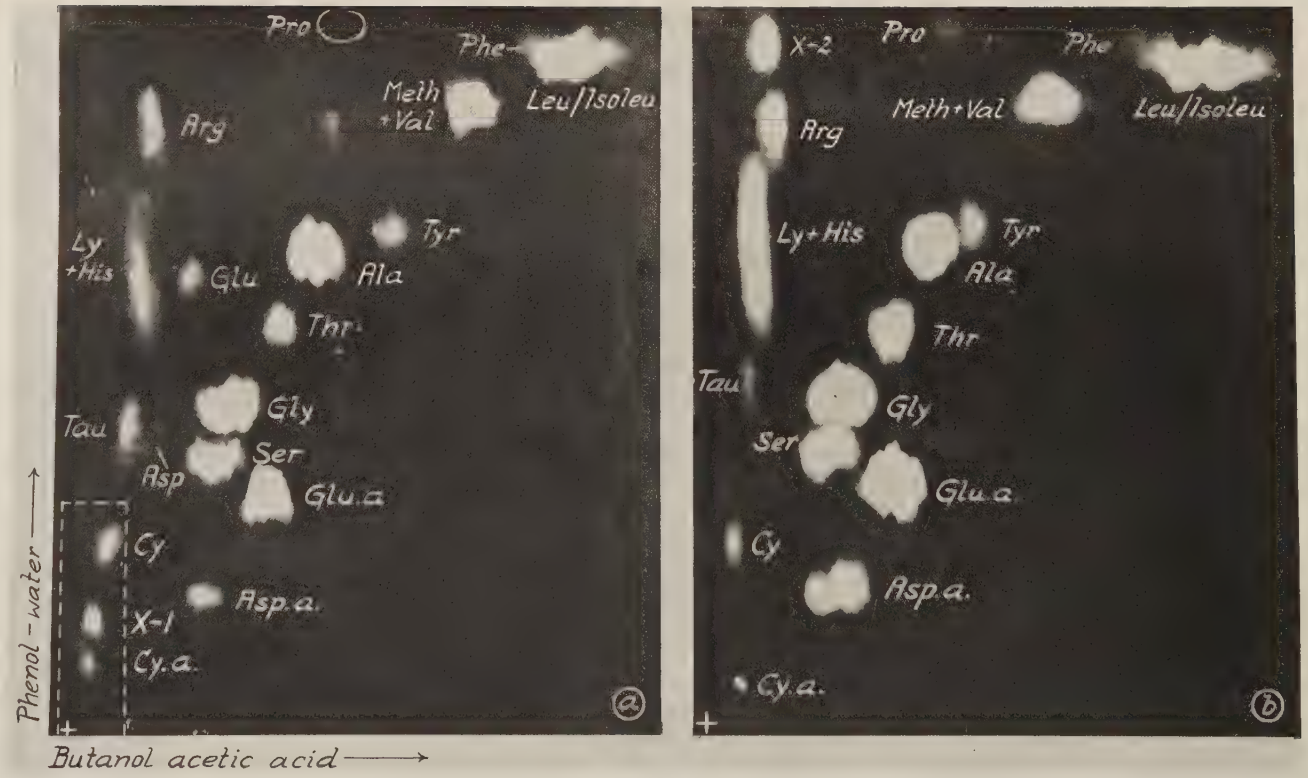

Fig. 3 Paper chromatograms of the ethanol-soluble fraction of samples prior to synchronous division (growth stage 3). 3a and 3b represent aliquots prior and after hydrolysis, respectively, corresponding to 6 mg dry weight each. Glu = glutamine, Asp = asparagine, other abbreviations as in figure 1.

D. Concentration of some sulphur containing compounds

The low concentration of the sulphur containing amino acids and the difficulty encountered in paper chromatography of these compounds were obstacles to quantitative determinations. However, the undoubtedly important role of these compounds in cellular metabolism prompted some preliminary ex-

TABLE 2

Amino acid composition of the ethanol-soluble fraction at various growth stages

1, exponential multiplication; 2, after end of heat treatment; 3, prior to synchronous division; 4, in the maximum stationary phase. Values are given in micromoles per 10 gram of dry weight.

AMINO ACID	GROWTH STAGE			
	1	2	3	4
(μM/g)				
Alanine	45.3	41.3	34.3	36.3
Arginine	31.5	27.0	38.2	30.1
Asparagine	7.9	7.3	6.2	6.9
Aspartic acid	17.3	21.6	27.1	18.8
Cystine	8.3	11.9	10.0	8.6
Glutamic acid	34.5	45.0	37.6	39.2
Glutamine	12.8	12.1	18.0	13.8
Glycine	33.4	29.6	26.8	29.9
Leucine/Isoleucine/Phenylalanine	34.0	48.4	60.3	46.0
Lysine/Histidine	35.8	24.4	29.8	28.4
Proline	12.1	11.3	9.9	10.6
Serine	18.7	18.3	21.8	19.1
Threonine	17.5	18.7	20.8	18.7
Tyrosine	14.1	20.2	16.2	17.2
Valine/Methionine	16.6	22.4	26.4	21.1

periments which are shown in figure 5. The amounts used for chromatography were increased three times (corresponding to 30 mg dry weight), and the time of development in both butanol and phenol was doubled (30 hours). The spots represent the compounds surrounded by the broken lines in figure 2. In figure 5 it can be seen that cysteic acid, X-1, and cystine show no marked changes during exponential multiplication (5a), after heat treatment (5b), or prior to the first synchronous division (5c). In the maximum stationary phase

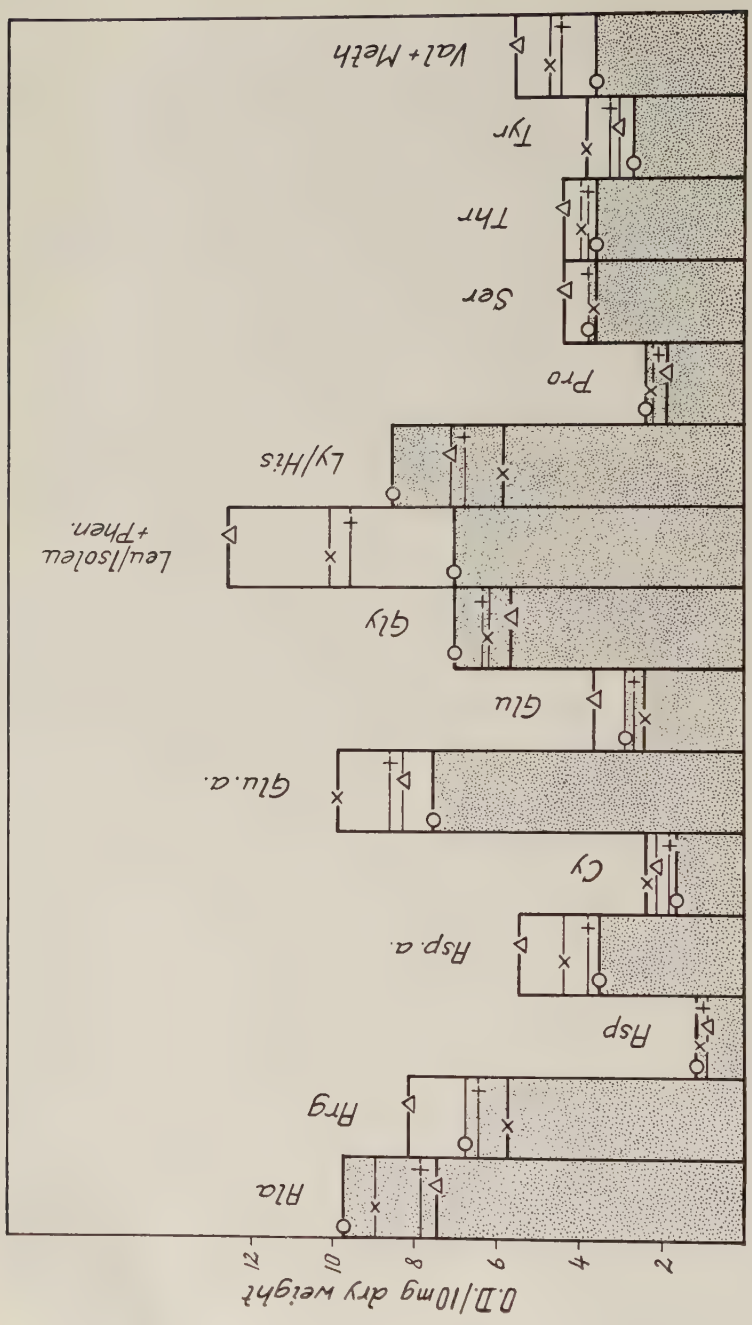

Fig. 4 Concentration of free amino acids at various stages of cell growth and cell division. The values on the ordinate give the total absorption, measured at 570 mμ (440 mμ for proline) for the amino acids listed above the histograms. The symbols in each histogram refer to the following growth stages: 1, exponential multiplication, (— O —); 2, after 7 heat shocks, (— X —); 3, prior to the first synchronous cell division (— Δ —); 4, approximately 18 hours later in the maximum stationary phase, (— + —). The dotted parts are values found for exponential growth.

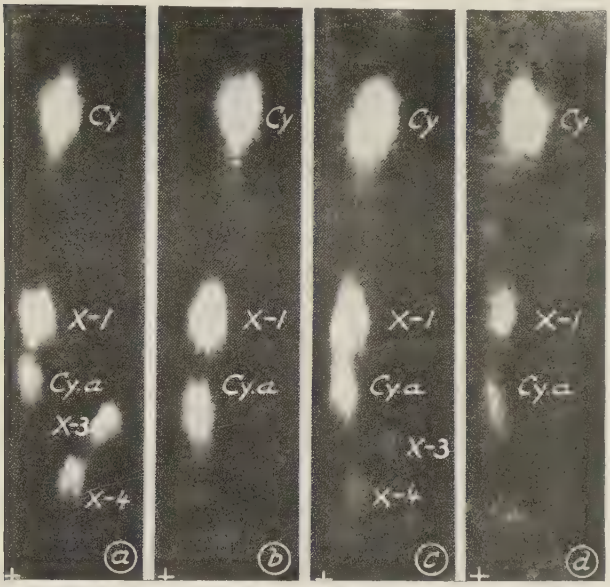

Fig. 5 Two dimensional paper chromatograms of some compounds in the unhydrolyzed samples of the ethanol-soluble fraction enclosed by the broken lines in figure 3a (a), exponentially growing culture; (b), after end of heat treatment; (c), prior to synchronous division; (d), maximum stationary phase.

(5d) the concentration of X-1 and cysteic acid seem somewhat reduced. Two unidentified spots (X-3, X-4) appear in the sample representing the log culture (5a). Traces of X-3 and X-4 could be found in (5c). Upon hydrolysis these two spots V and X-1 disappear, suggesting their probable peptide nature.

E. Comparison of free and protein amino-acid fraction

The free amino acid fraction was compared with the total amino acid content on the basis of absorption measurements at 570 mμ of the color developed with ninhydrin. The results of 4 experiments range from 12.6 to 15% with an average of 13%, for the free amino acid content.

DISCUSSION

A. Errors of analysis

The various growth steps are designated by the time at which the samples were removed from the experimental cul-

ture. This does not mean, however, that the metabolic pattern of the cells could be fixed at the moment of removal. The necessary centrifugation and washing of the cells caused a delay of about 25 minutes, until they could be fixed by heating. This delay is probably insignificant when cells in the logarithmic or stationary phase of growth are handled. However, accurate timing of the samples after treatment and in the predivision phase is obviously difficult and impossible with the present methods.

Errors pertaining to the analytical procedure, the development of the color with ninhydrin and elution and spectrophotometric determination of the color are probably very small. The standard deviation for the individual reading is $\pm$ 0.4% (Connell et al., '51). The error in the analyses reported here was estimated from twelve duplicates, and the standard deviation (SD) was 8.5%.

B. Terminology

For the sake of convenience we speak of a free amino acid fraction. It would, however, be more accurate to refer to a "fraction, soluble in 80% ethanol" and thus indicate that smaller peptides may also be found in this fraction. The "peptide protein fraction" means the "fraction precipitable in 80% ethanol."

C. Comparison of the results with other findings

The amounts of the amino acids isolated are comparable to the pattern reported for *Tetrahymena pyriformis* E (Wu and Hogg, '52). On a molar basis the most abundant free amino acids in both strains are glutamic acid, alanine, arginine and glycine, but leucine, isoleucine, phenylalanine, lysine and histidine cannot be compared since we could not separate these amino acids completely. Similarly, glutamic acid, aspartic acid, glycine and alanine are predominant in the composition of the cellular protein of both strains. The total nitrogen of the cells was found to be 7.8% of the dry weight (Wu and

Hogg, '52). This value remained remarkably constant when cells of strain E were cultivated in different media, and it might therefore be used to express the data on the basis of percentage of the total amino acid nitrogen of the cells. Both strains have relatively low concentrations of sulphur containing amino acids.

The possible significance of changes of free and protein-SH groups prior and during cell division has been repeatedly emphasized (Rapkine, '31; Chalkley, '37; Mazia, '54) and our data show some changes in the concentration of sulphur containing amino acids. However, these changes could not be attributed to the physiological conditions for cell growth and cell division. Furthermore, the methods used did not permit us to distinguish between the oxidized and reduced forms of these compounds. Therefore, we intend to continue these investigations in order to obtain better qualitative and quantitative data on sulphur containing amino acids in both the free and peptide forms at the various growth stages.

SUMMARY

1. Mass cultures of *Tetrahymena pyriformis GL* were sampled in 4 characteristic growth stages: (a) during normal exponential multiplication when 5 to 8% of the cells are in division. (b) After the end of a standard temperature-cycle treatment permitting cell growth but not cell division. (c) Immediately prior to the synchronous division in which 80 to 85% of the cell population undergoes simultaneous multiplication. (d) In the maximum stationary phase in which cell size is reduced to 50% of the values found in the early logarithmic phase of growth and where there is practically no cell division.

2. With help of paper chromatography and ninhydrin reactions, free amino acids and amino acids of peptides and proteins were quantitatively estimated.

3. The pattern of the protein amino acids shows a remarkable stability at the various growth stages analyzed (S = 6.1%).

4. Greater variability was encountered in the free amino acid pattern (SD = 16.9%). This variability exists not only within but also between experiments. The three fold increase in cell volume during treatment, as well as the significant size reduction in the maximum stationary phase, is not reflected in the general pattern of the free amino acid pool. Therefore, cessation of growth in the maximum stationary phase is apparently not caused by a depletion of any of the amino acids analyzed.

5. The free amino acid fraction is 12.6 to 15% of the total amino acid content when the ninhydrin colors are compared at 570 mμ.

6. The relative proportions of the free amino acids bore no resemblance to those of the protein fraction.

7. Glutamine, asparagine and an unidentified compound (X-1, presumably glutathione) disappear from the ethanol soluble fraction upon hydrolysis. Under these conditions a new spot appears near arginine (X-2). A marked increase in strength of the spots given by aspartic acid, glutamic acid, glycine and threonine occur upon acid hydrolysis.

8. Qualitative analyses for sulphur containing compounds show some changes which could not, however, be attributed to the physiological conditions for cell growth and cell division. The significance of these changes is not yet understood.

ACKNOWLEDGMENTS

The authors would like to thank Dr. D. Atkinson of the Department of Biochemistry, UCLA and Dr. R. Schaffer, University of Southern California, for their helpful suggestions in course of this work. Mr. A. Louderback rendered much skillful technical assistance.

LITERATURE CITED

Awapara, J. 1948 Application of paper chromatography to the estimation of free amino acids in tissues. Arch. Biochem., *19:* 172.

Chalkley, H. W. 1937 The chemistry of cell division. VII. Distribution of SH in ameba proteus in interkinesis and mitosis as shown by the nitroprusside test. Protoplasma, *28:* 489.

CONNELL, G. E., G. H. DIXON AND CH. S. HANES 1955 Quantitative chromatographic methods for the study of enzymic transpeptidation reactions. Canad. J. Biochem. Physiol., *33:* 416.

HOLZ, G. G., O. H. SCHERBAUM AND N. WILLIAMS 1957 The arrest of mitosis and stomatogenesis during temperature induction of synchronous division in Tetrahymena pyriformis, mating type 1, variety 1. Exp. Cell. Res., *13:* 618.

KAVANAU, J. L. 1954 Amino acid metabolism in the early development of the sea urchin Pacentrotus lividus. Ibid., *7:* 530.

KIDDER, G. W., AND V. C. DEWEY 1951 The biochemistry of ciliates in pure culture. In: Biochemistry and Physiology of Protozoa, ed. A. Lwoff, p. 324. Academic Press, New York.

LYTTLE, R. J., AND E. D. MOFFAT 1957 Identification of amino acids on paper chromatograms using a polychromatic indicator. Research Project Report N.M. 520604.4.1, Navy Department, Wash. 25, D.C.

MAZIA, D. 1954 SH and Growth in Glutathione. Academic Press, New York.

ORMSBEE, R. A. 1942 The normal growth and respiration of Tetrahymena geleii. Biol. Bull., *82:* 423.

RAPKINE, L. 1931 Sur les processus chimiques ou cours de la division cellulaire. Annales de physiol. physicochemie biol., *7:* 382.

SCHERBAUM, O. 1956 Cell growth in normal and synchronously dividing mass cultures of Tetrahymena pyriformis. Exp. Cell. Res., *11:* 464.

———— 1957a Studies on the mechanism of synchronous cell division in Tetrahymena pyriformis. Ibid., *13:* 11.

———— 1957b The content and composition of nucleic acids in normal and synchronously dividing mass cultures of Tetrahymena pyriformis. Ibid., *13:* 24.

———— 1957c The application of a standard counting method in estimation of growth in normal and heat treated cultures of Tetrahymena pyriformis. Acta path. et microbiol. scand., *40:* 7.

———— 1957d The division index and multiplication in a mass culture of Tetrahymena following inoculation. J. Protozool., *4:* 257.

SCHERBAUM, O., AND E. ZEUTHEN 1954 Induction of synchronous cell division in mass cultures of Tetrahymena pyriformis. Exp. Cell Res., *6:* 221.

———— 1955 Temperature induced synchronous divisions in the ciliate protozoan Tetrahymena pyriformis in synthetic and proteosepeptone media. Exp. Cell Res., Suppl. *3:* 312.

TOENNIS, G., AND J. J. KOLB 1951 Techniques and reagents for paper chromatography. Analyt. Chem., *23:* 823.

WELLINGTON, E. F. 1951 Amino acids of two insect viruses. Biochem. et Biophys. Acta, *7:* 238.

WU, C., AND J. F. HOGG 1952 The amino acid composition and nitrogen metabolism of Tetrahymena geleii. J. Biol. Chem., *198:* 753.

———— 1956 Free and nonprotein amino acids of Tetrahymena pyriformis. Arch. Biochem. Biophys., *62:* 70.

COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

CONTRACTILE RESPONSES TO ELECTRICAL STIMULATION OF GLIAL CELLS FROM THE MAMMALIAN CENTRAL NERVOUS SYSTEM CULTIVATED IN VITRO [1]

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Galveston

ONE FIGURE

It has been shown that astrocytes cultivated *in vitro* respond to electrical stimulation by a transient reduction of their resting potential and subsequent recovery (Hild, Chang and Tasaki, '58). Time-lapse cinematographic studies reveal that astrocytes under *in vitro* conditions frequently show spon-

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taneous contractions of their cell bodies (Hild, '54) similar to the contractions of oligodendrocytes (Canti, Bland and Russell, '35; Pomerat, '51). It seemed of interest to investigate whether such contractions could be produced by electrical stimulation and, if so, whether the threshold required would be of the same order of magnitude as that necessary for the evocation of an electrical response.

Glial cells from the cerebellum of kittens and puppies were grown according to our standard methods (see Hild, '57) on 12 × 50 mm coverslips in roller tubes. They were maintained for periods of from 4 to 36 days before they were used for experiments. The coverslips on which the cells were cultivated were mounted on an oblong chamber 4 mm in depth with a microscope slide as its bottom. One long side of this chamber was left open for filling the chamber with Gey's balanced salt solution and for introducing the stimulating electrodes while the other three sides were sealed with beeswax. The chamber was placed on a modified microscope stage and the whole assembly was enclosed in a moist chamber with openings for the stimulating wires and the microscope objective. Both the microscope and the micromanipulators holding the stimulating electrodes were placed in an incubator maintained at 35 ± 0.5°C. Time-lapse motion picture records were taken on 16-mm film at a rate of 8 frames per minute. The cells were observed and recorded with Zeiss phase-contrast optics, using 25, 40 and 63× objectives and a 10× ocular.

Rectangular stimulating pulses from a Grass stimulator were delivered to the cells through a small glass capillary electrode filled with Gey's balanced salt solution. A large indifferent electrode (stimulus anode) was connected to the fluid in the chamber at some distance away from the culture by means of an agar bridge. The stimulating electrodes were isolated from ground by the use of a stimulus isolation unit. The tip of the stimulating electrode was placed usually at a distance of from 4 to 60 μ from the cells under investigation with the aid of a Leitz micromanipulator. The tip diameters

of the electrode ranged from 15 to 85 μ and the resistance varied between 0.2–3.0 megohms.

After choosing suitable fields a base line or control sequence of film was taken in order to determine whether or not any spontaneous contractions occurred among the cells in the field. This also served as a guide to obtain information concerning the general behavior of the cells in particular fields. In each experiment, a series of stimuli was applied at 15–20 minute intervals starting with a single pulse of low intensity and gradually increasing at each step in intensity, duration or number of pulses. The duration of the individual stimulating pulse was varied between 5 and 20 msec. and its amplitude between 20 and 150 volts. The repetition frequency of the pulses from the stimulator was set at either one or 10 per sec. and the stimulator switch was closed for a period of 1 to 10 sec.

As the intensity of the stimulus reached the threshold of a particular cell, a very weak contraction was noticeable in response to a single stimulating pulse. This contraction became stronger when the intensity was increased, but beyond a certain point further increase of stimulus intensity did not produce any greater contraction. After a normal contraction in response to a stimulus, the cells expanded again to their previous size and resumed normal activity (protoplasmic movements, membrane undulations, etc.). However, if the intensity became excessively strong the cells were irreversibly damaged.

Although not all parts of a cell would contract simultaneously, the duration of the contraction phase as determined by film analysis on the response of the main body of the cell ranged from 1.4–3.4 minutes with an average of 2.8 minutes, whereas the relaxation phase had a range of 6–16 minutes averaging about 11 minutes. Such activities were best observed in the case of well-isolated astrocytes (fig. 1). When the stimulating current was applied to a larger network of cells as seen at the margin of the explants proper, the whole mass of cells with their intertwined processes showed con-

certed contraction and expansion which extended beyond the field of observation. In some instances oligodendrocytes were also seen to respond to stimulation with contractile activity.

Sometimes a latent period of about 1.5–4 minutes was observed between the time of stimulation and the start of the active contraction of a given cell. This was only seen when the cells were more than approximately 60 μ from the tip of the stimulating electrode.

It was noticeable that not all cells in a given field responded to the same stimulus. Some cells remained completely unaffected, whereas others showed very distinct responses. This phenomenon was not correlated with the relative distances of the cells from the electrode. The age of the cultures seemed to have some influence on the contractile activity of the glial cells. As the cultures grew older, an increasing proportion of cells lost their spontaneous contractile activity. From such cells only slight or no mechanical response could be obtained. This probably accounts for a large percentage of the negative results when such cultures inadvertently were used. There remains, however, a certain number of cells which, despite their normal activity and appearance, did not respond to stimulation. This perhaps may be due to certain inherent properties of the cells involved, such as, for example, a high membrane conductance at the time of stimulation.

The time courses of the electrical and the contractile responses showed a marked difference in their orders of magnitude, the electrical responses lasting 4–6 seconds and the contractile responses about 13 minutes. The current densities

Fig. 1 Three selected film frames of a motion picture sequence showing the effect of electrical stimulation on an isolated astrocyte. Eight-day-old culture of kitten cerebellum. Phase contrast. The bar in the upper left corner of a represents 50 μ length. Stimulating electrode with a tip diameter of approximately 12 μ; distance from the cell body approximately 24 μ; a, immediately before stimulation. The cell body shows normal size and configuration. b, 1.7 minutes after stimulation. The cell body is markedly contracted and the processes slightly thickened due to shifting of cytoplasm from the cell body into the processes. c, 7.5 minutes after stimulation. The cell body has expanded again and regained its original configuration. The total duration of this contractile response was 7.5 minutes which represents the shortest duration observed in the course of this investigation.

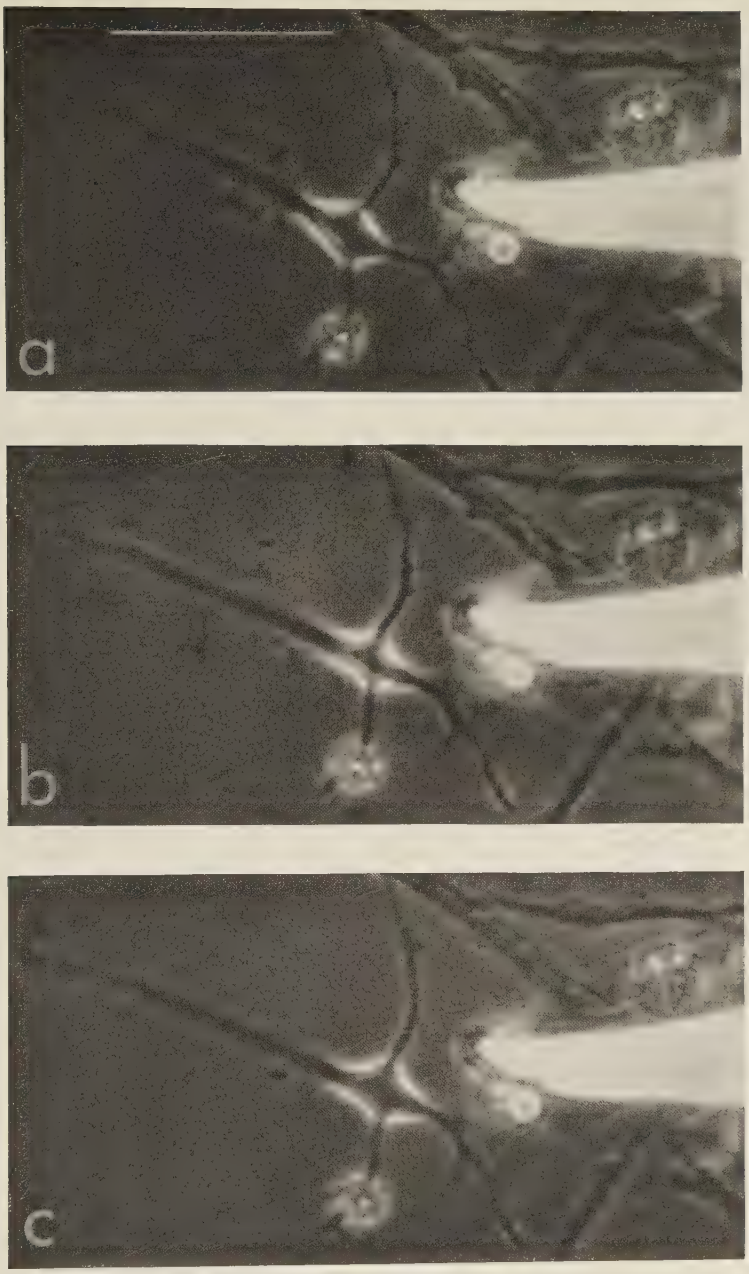

Figure 1

required for electrical and contractile responses are found to be of the same order of magnitude within a narrow range. This leads us to the conclusion that the two responses are closely related. Thus, the electrical response precedes the contractile response and may very well be the sign of the beginning of a sequence of events leading to the contraction. It is also interesting to note that both the electrical and the contractile response gradually increase with increasing intensity of the stimuli and, therefore, do not show any "all-or-none" character.

However, the possibility cannot be excluded that in some cells the electrical response is not followed by a contractile response. To elucidate this point, it would be necessary to record simultaneously with an oscilloscope over an intracellular electrode and time-lapse cinematography.

ACKNOWLEDGMENT

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LITERATURE CITED

CANTI, R. G., J. O. W. BLAND AND D. S. RUSSELL 1935 Tissue culture of gliomata. Cinematograph demonstration. Proc. Assoc. Res. Nerv. Ment. Dis., *16:* 1–24.

———— 1937 Tumors of the Nervous System. Williams and Wilkins, Baltimore.

HILD, W. 1954 Das morphologische, kinetische und endokrinologische Verhalten von hypothalamischem und neurohypophysärem Gewebe in vitro. Z. Zellforsch., *40:* 257–312.

———— 1957 Observations on neurons and neuroglia from the area of the mesencephalic fifth nucleus of the cat in vitro. Ibid., *47:* 127–146.

HILD, W., J. J. CHANG AND I. TASAKI 1958 Electrical responses of astrocytic glia from the mammalian central nervous system cultivated in vitro. Experientia, *14:* (June issue.)

POMERAT, C. M. 1951 Pulsatile activity of cells from the human brain in tissue culture. J. Nerv. Dis., *114:* 430–449.

EFFECTS OF OVULATION UPON THE GLYCOGEN CONTENT OF THE FROG OVIDUCT [1]

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The physiology of the amphibian oviduct has not been investigated sufficiently to give an understanding of the metabolism taking place during the secretion of the jelly membranes.

Although the amphibian oviduct has been well investigated morphologically (Lebrun, 1891 and Miyauchi, '39), a search of the literature failed to reveal information regarding its quantitative glycogen content. Ohohashi ('24) determined the presence of glycogen in the oviduct of the frog and toad during all seasons of the year but cited no actual values. Several workers have studied the glycogen content of many tissues of various Amphibia (Athanasiu, 1899; Bleibtreu, '10; Kato, '10; Goldfederova, '26; Wertheimer, '28). Bleibtreu, working on *Rana fusca* and *Rana esculenta,* studied the glycogen content throughout the year in the liver, muscle, skin, central nervous system, and ovary. He observed the glycogen content of the ovary to be at its lowest level in August, after which it rose to a maximum in April at the time of egg-laying. He found that the glycogen content of the liver at the time of egg-laying was almost zero, and postulated that the increase in the ovaries was largely at the expense of the liver. He did not determine the glycogen level of the oviduct, which appears to have a relatively high glycogen content. Wert-

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heimer ('28) confirmed the findings of Bleibtreu. He reported the ovarian glycogen was not affected by body metabolism nor by agents such as strychnine, adrenaline, and insulin which provoke glycogenolysis in liver, muscle, and heart tissue. Needham ('31) called this glycogen of the ovary "reproductive" glycogen. It would be of interest to know if the glycogen of the oviduct responds in a manner similar to this "reproductive" glycogen of the ovary.

The purpose of the present study was to investigate changes in the carbohydrate metabolism of the frog oviduct subsequent to induced ovulation. The passage of eggs through the oviduct and the secretion of the jelly membranes are metabolic processes and, consequently, changes in the chemical structure of the oviduct should be expected.

METHODS AND MATERIALS

Female frogs *(Rana pipiens)*, weighing between 75 and 100 gm, were removed from refrigerator hibernation and treated as follows. The experimental animals were divided into three groups. The first group was anesthetized with sodium pentobarbital (20 mg/kg) injected into the dorsal lymph sac. A small incision was made on the ventral surface, lateral to the ventral abdominal vein, and both oviducts were ligated approximately 2 cm below the ostium. The peritoneal and body wall incisions were sutured and the frogs allowed 48 hours recovery time. The second group was injected intraperitoneally with a solution containing macerated pituitaries from 8 small frogs (mixed sex) of the same species. These animals were not anesthetized nor were the oviducts tied off. A third group was treated as per group 1, relative to the ligation of the oviducts, and group 2, with reference to the injection of macerated pituitaries. The control group of amphibians was removed from refrigeration along with the experimental groups. After injection of the pituitary solution the frogs were allowed to remain in a moist environment at room temperature for $\pm$ 60 hours. The animals were

then killed and a section of each oviduct was removed for glycogen analysis.

Miyauchi ('39) functionally divided the frog oviduct into 4 parts: (1) pars recta, (2) upper pars convoluta, (3) lower pars convoluta and (4) uterus. In the present investigation a cephalad portion of the lower pars convoluta, weighing on an average of 250 mg, was sampled for analysis of the glycogen. This particular portion of the oviduct was selected because Miyauchi found that all three zones of the jelly membrane surrounding the egg are secreted from the pars convoluta.

A piece of tissue from both the right and left oviducts was analyzed for acid-soluble and acid-insoluble glycogen. The acid-soluble glycogen fraction is variously referred to in the literature as the lyo-, free, acid-extractable, or labile glycogen while the acid-insoluble fraction is termed the desmo-, bound, fixed, residual, or stable glycogen component. The acid-soluble fraction was determined by a method suggested by Bloom, Lewis, Schumpert, and Shen ('51). The acid-insoluble form was determined by a method previously described (Meyer, Russell, Platner, Purdy and Westfall, '55). Acid hydrolysis of both fractions was obtained with sulfuric acid and the resulting glucose was then quantitatively measured by the anthrone procedure as described by Seifter, Dayton, Novic and Muntwyler, ('50). A Beckman model B spectrophotometer was used for all colorimetric determinations.

RESULTS

The determination of glycogen fractions in the oviduct of the hibernating *Rana pipiens* is indicative of the glycogen content of the oviduct in the breeding condition since this particular species is in the breeding state with the onset of hibernation.

The glycogen changes following the three experimental procedures are given in table 1. The changes observed in the acid-insoluble glycogen fraction were statistically compared by simple analysis of variance and the "t" test. The F value

was 2.46 giving a level of significance of > 0.05. Similar statistics were applied to the acid-soluble glycogen of the control and experimental groups. The F value was 24.97 and the level of significance was < 0.001. All groups were significantly different except two and three. The effects of ligation were determined in the first series of animals (group 1). This ligation was responsible for a 22% decrease in the acid-soluble glycogen. In group 2 there was a 49% loss in the acid-soluble glycogen fraction following artificially induced ovulation. This

TABLE 1

The effects of oviduct ligation and artificially induced ovulation on glycogen fractions in the oviduct of the frog (R. pipiens)

GROUP	CONDITION	NO. OF ANIMALS	GLYCOGEN (MG/100 GM)		
			Acid-insoluble	Acid-soluble	Total
Control		22	55 ± 3.2 [1]	279 ± 18.8 [1]	334
1	Oviducts ligated, not injected	7	54 ± 5.2	208 ± 17.7	262
2	Oviducts not ligated, injected	28	50 ± 3.1	142 ± 8.5	192
3	Oviducts ligated, injected	13	41 ± 3.7	132 ± 10.2	173

[1] Standard error of the mean.

could be attributed to either the action of the pituitary hormones and/or to the passage of eggs through the oviduct. The effect of the hypophyseal hormones alone was determined as the result of oviduct ligation, preventing passage of eggs through the oviduct (group 3).

DISCUSSION

The results obtained in the present paper indicate that of the 53% decrease in the acid-soluble glycogen form, 27% is due to the effects of the hypophyseal hormones. The effects produced as the result of surgical ligation are responsible for the difference. The loss of acid-soluble glycogen, due to the action of the pituitary hormones (27%), less the absolute

decrease in group 2 (49%), may be accounted for by oviduct activity during passage of the eggs.

Galli Mainini ('50) postulated that the stimulus for secretions from the oviduct *(Bufo arenarum),* following induced ovulation by a pituitary injection, was a function of activated ovarian tissue. The hormonal injection, consequently, had a direct and specific effect upon ovarian tissue but no independent action upon the oviduct. This supposition was supported by the fact that extracts obtained from ovaries during induced ovulation initiated a secretory action when injected into oviducts of other toads. Kambara ('56), employing histochemical techniques on the oviduct of the Japanese newt *(Triturus pyrrhogaster),* offers some opposition to the fact that the oviduct contains glycogen. He suggests the staining reaction is due to the presence of mucopolysaccharides. In the present paper acid-insoluble glycogen is obtained by hot alkaline digestion and alcoholic precipitation and cannot, therefore, be assumed to be a mucopolysaccharide. The acid-soluble precipitate also was subjected to hot alkaline digestion and precipitation with ethanol and compared to an aliquot of the same tissue extracted with cold 10% trichloroacetic acid and cold ethanol. The glycogen changes observed after the alkaline extraction are not significant. This also was confirmed by using the same procedures with rat uterus and liver.

The data suggests there is a significant decrease in the acid-soluble glycogen component due to the ligation of the oviduct, the action of pituitary hormones upon the oviduct (via the ovaries, perhaps), and the passage of eggs through the oviduct. If the glycogen loss is related to the egg jelly secretion it is probable that several intermediary substances are involved. Folkes, Grant, and Jones ('50), investigating the chemical constituents of frog egg jelly, reported the presence of several hexoses: glucose, galactose, mannose, fucose, and xylose; two hexosamines, glucosamine and chondrosamine; and 18 amino acids. Minganti ('55) reported various combinations of monosaccharide residues, i.e., glucose, galactose, mannose, and fucose, in the jelly membranes of 4

species of Amphibia: *Rana esculenta, Bufo vulgaris, Discoglossus pictus,* and axolotl. Glucose, however, was found only in the egg jelly of *Discoglossus.* He determined these monosaccharide residues as hexoses and hexosamines. A quantitative determination of the egg jelly of *Bufo vulgaris* gave the following: proteins, 37.8%; hexosamines, 40.4%; fucose, 10.4%; and galactose and mannose, 11.4%. Lison ('53) found that the mucoids of the frog oviduct contained galactose and acetylglucosamine.

On the basis of these reported chemical substances comprising the jelly membranes, glucose appears principally as a hexosamine while galactose, mannose, and fucose are the predominant monosaccharides. If glycogen is utilized during the secretory processes it is not used principally as glucose. The latter must be converted to glucosamine, other hexosamines and hexoses, and finally to mucopolysaccharides. Dorfman ('55) suggests the following metabolic pathway in the formation of hexosamines from glycogen: glycogen → glucose-1-phosphate → glucose-6-phosphate → glucosamine-6-phosphate → glucosamine.

SUMMARY

The changes in the two glycogen fractions of the oviduct of the frog *(Rana pipiens)* were investigated subsequent to various experimental procedures.

The more stable, acid-insoluble glycogen fraction was not significantly altered. Induced ovulation resulted in a 49% decrease in the acid-soluble glycogen component. This decrease was due to the action of the pituitary hormones and the passage of the eggs through the oviduct. The effect of the hypophyseal hormones alone accounted for 27% of the loss. This was determined by ligation of the oviduct followed by induced ovulation. The effects of surgical ligation also were responsible for a significant decrease in this labile glycogen form.

It is suggested that glycogen is not used primarily as glucose in the formation of the jelly membranes from mucopolysaccharides. The glucose derived from glycogen, however, probably is utilized in the metabolic pathway.

LITERATURE CITED

ATHANASIU, J. 1899 Über den Gehalt des Froschkörpers an Glykogen. Arch. f. d. ges. Physiol., *74:* 561–569.

BLEIBTREU, M. 1910 Glykogen im Froscheierstock. Ibid., *132:* 580–599.

BLOOM, W. L., G. T. LEWIS, M. Z. SCHUMPERT AND T. SHEN 1951 Glycogen fractions of liver and muscle. J. Biol. Chem., *188:* 631–636.

DORFMAN, A. 1955 Metabolism of the mucopolysaccharides of connective tissue. Pharmacol. Rev., *7:* 1–31.

FOLKES, B. F., R. A. GRANT AND J. K. N. JONES 1950 Frog-spawn mucin. J. Chem. Soc., *8:* 2136–2140.

GALLI MAININI, C. 1950 Secreción del oviducto del sapo por el ovario en ovulación. Rev. Soc. Argent. Biol., *26:* 166–178.

GOLDFEDEROVA, A. 1926 Le glycogène au cours de l'ontogènése de la grenouille et sous l'influence des saisons. C. R. Soc. Biol., *95:* 801–804.

KAMBARA, S. 1956 Histochemical studies on the distribution of mucopolysaccharides in the oviduct of the newt, *Triturus pyrrhogaster.* Annot. Zool. Japon., *29:* 146–150.

KATO, K. 1910 Über das Verhalten des Glykogenes im Eierstocke der Frösche zu den verschiedenen Jahreszeiten. Arch. f. d. ges. Physiol., *132:* 545–579.

LEBRUN, H. 1891 Recherches sur l'appareil génital female de quelques batraciens indigenes. La Cellule, *7:* 415–484.

LISON, L. 1953 Histochimie et Cytochimie Animales. Gauthier-Villars, Paris, 2nd. ed., 607 pp.

MEYER, D. K., R. L. RUSSELL, W. S. PLATNER, F. A. PURDY AND B. A. WESTFALL 1955 Synthesis of glycogen fractions by heart homogenates. Proc. Soc. Exp. Biol. Med., *90:* 15–17.

MINGANTI, A. 1955 Chemical investigations on amphibian egg jellies. Exp. Cell Res., (Suppl.) *3:* 248–251.

MIYAUCHI, T. 1939 Untersuchungen über Bau und Funktion des Eileiters von *Rana.* J. Sci., Hirosima Univ., Series B, Div. 1 (Zool.), *6:* 79–102.

NEEDHAM, J. 1931 Chemical Embryology. Cambridge University Press, London. 3 vols., 2021 pp.

OHOHASHI, Y. 1924 Comparison of the distributions of glycogen in mammals and anura (Rana nigromaculata et Bufo bufo japonicus). Japan Med. World, *4:* 64–68.

SEIFTER, S., S. DAYTON, B. NOVIC AND E. MUNTWYLER 1950 The estimation of glycogen with the anthrone reagent. Arch. Biochem., *25:* 191–200.

WERTHEIMER, E. 1928 Stoffwechsel regulationen. XI. Mitteilung. Über die Sonderstellung der Eizelle im Stoffverbraunch. Arch. f. d. ges. Physiol., *221:* 119–138.

COMPOSITION OF BOVINE AMNIOTIC AND ALLANTOIC FLUIDS USED AS CELL CULTURE MEDIUM [1]

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ONE FIGURE

Various workers have now utilized the fluids of the bovine embryo as a convenient and satisfactory primary constituent of media for tissue culture. Enders ('53) first reported the use of bovine amniotic fluid for culture of human tissues as applied to virus cultivation. Subsequently, Malherbe ('54) noted that embryonic fluids had been employed earlier by others for certain *in vitro* culture studies. He also pointed out that fluid for culture purposes collected from gravid bovine uteri by blind puncture might contain either amniotic or allantoic fluid, or both, and concluded that amniotic fluid was more satisfactory for growth of human tissues.

Although some information is available concerning composition of bovine amniotic and allantoic fluids, few data appear in the literature relative to fluids collected at those gestational ages customarily chosen for tissue culture purposes. Earlier studies (Döderlein, 1890; Gürber and Grunbaum, '04; and Paton, Watson, and Kerr, '07) dealt mainly

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with possible origin, alterations in volume, and physical characteristics of amniotic and allantoic fluids during gestation. Chemical determinations reported, primarily on fluids from older embryos, showed great differences in values. The striking variation in chemical composition of embryonic fluids is also apparent in more recent reports for the sheep (Malan, Malan, and Curson, '37), and for bovine embryos of unspecified size (Collip, '27), or near term (Conklin et al., '31).

The paucity of available information concerning composition of embryonic fluids of young bovine embryos as used for tissue culture prompted the following studies. We also encountered remarkable variation in the values of many components studied. It should be noted that results obtained pertain to the composition of embryonic fluids as customarily used for tissue culture purposes, and do not necessarily represent *in vivo* conditions.

MATERIALS AND METHODS

Intact gravid bovine uteri were brought directly to the laboratory from the local abattoir. Within two to 6 hours after removal from the cow, the surfaces of uteri were cleansed and opened so that amniotic and allantoic fluids could be removed from their respective sacs under direct vision. However, as noted below, some specimens of fluid were collected by "blind" puncture and drainage of the suspended uterus with a trocar. Aliquots of all fluids were centrifuged at approximately 1500 r.p.m. for 10 minutes, and supernates stored frozen at –15°C for subsequent chemical determinations. Direct cell counts were made with a hemocytometer on some fluids before centrifugation. Fluids titrated for buffering capacity consisted of decanted supernates from specimens kept overnight in the refrigerator (5°C) in tightly stoppered flasks. Fluids which showed gross particulate matter or red cells were discarded. In addition, samples of sterile bovine amniotic fluid were obtained from a commercial source.[3] Modified Medium 199 (Salk, Youngner, and

[3] Obtained from Cudahy Packing Company, Omaha, Nebraska.

Ward, '54) was freshly prepared and used with addition of bicarbonate as noted for chemical determinations and titration of buffering capacity. The methods employed for biochemical determinations were those in regular use for clinical laboratory work: protein (Ayer, Dailey, and Fremont-Smith, '31), NPN (Peters and Van Slyke, Chap. X, '32), chloride (Peters and Van Slyke, Chap. XXX, '32), phosphorus (Fiske and Subbarow, '25), calcium (Tisdall, '23), and CO_2 and bicarbonate (Van Slyke and Neill, '24). Sodium and potassium were measured with Barkeley flame photometer. Aliquots of fluids for CO_2 and bicarbonate determinations were overlaid with sterile mineral oil as soon as collected and stored in the refrigerator until tested. All determinations were done in duplicate and good agreement in results was obtained. Samples of fluids were titrated with 1 N HCl for buffering capacity in 25-ml volumes using a Model H Beckman pH meter with a glass electrode. Duplicate or triplicate titrations were run for each specimen, and the result expressed as the average. Individual titrations were completed within 10 minutes.

RESULTS

Cell counts. Cells were enumerated from amniotic and allantoic fluids of 6 embryos ranging from 6 to 18 cm in crown-rump length. Counts of 0 to 270 red cells/mm^3 were encountered, but most counts were 4–45/mm^3. Other cells consisted mainly of leukocytes and epithelial cells in total numbers usually 10–20/mm^3, although one allantoic fluid had 180/mm^3. No association of particular cell type with either amniotic or allantoic fluid was noted.

Biochemical determinations. Amniotic and allantoic fluids from 13 gravid bovine uteri were separately tested for various constituents, and similar determinations were done on fluids obtained by blind trocar drainage from 6 additional specimens. This latter method is usually employed in harvesting such fluids for tissue culture work. The embryos studied ranged from 4.5 to 26 cm in crown-rump length. Chemical determinations for amniotic as compared to allantoic fluids

TABLE 1

Biochemical determinations on bovine amniotic and allantoic fluids

EMBRYO CROWN-RUMP LENGTH [1]	NON-PROT. N_2		PHOSPHORUS		CALCIUM		SODIUM		CHLORIDE		POTASSIUM		CO_2	
	Amn.	All.	Amn.	All.	Amn.	All.	Amn.	All.	Amn.	All.	Amn.	All.	Amn.	All.
cm	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*m Eq./l*	*m Eq./l*	*m Eq./l*	*m Eq./l*	*m Eq./l*	*m Eq./l*	*m Eq./l*	*m Eq/l*
4.5	17	41	2.6	1.8	6.6	17.0	130	103	122	79	15.5	1.3		
7.0			3.9	6.4			136	108	117	61	12.8	4.3	27.5	32.5
7.5													23.8	26.4
9.0	22	59	1.8	1.5	5.2	27.6	131	75	118	58	15.7	3.4		
10.0	14	46	2.2	4.6	5.4	15.4	136	108	125	80	14.3	4.9		
11.0													23.1	28.8
14.0	16	147	1.1	25.4	4.1	52.8	128	30	121	11	18.8	11.8		
14.5			1.1				130		118		18.2		7.9	
17.0			1.0	26.7			66	32	125	18	18.6	15.0	24.1	6.0
20.0	14	156	0.7	39.6	3.6	5.8	133	14	124	6	20.5	68.3		
20.5													24.9	25.6
24.0	147	88	1.8	6.4	5.1	23.7	126	12	124	7	16.1	2.7		
24.0	40	70	3.9	5.1	7.0	8.6	106	104	103	95	21.8	21.6		
Aver.	39	87	2.0	13.0	5.3	21.6	122	65	120	46	17.2	14.8	21.9	23.9
Stand. dev.	45	43	1.1	13.0	1.1	14.6	20	40	6	34	2.7	19.9	6.4	9.2

[1] Embryos of this range in size would represent approximate weights of 7 to 325 grams, or gestational ages of about 50 to 100 days (Swett, Matthews, and Fohrman, '48).

are summarized in table 1. Extreme variation in values for many of the chemical constituents tested is apparent, and no particular trend is evident with increasing embryo size. Phosphorus, calcium, chloride, and potassium values in amniotic fluids were relatively constant. Amniotic fluids generally showed less variation for all items tested than did allantoic fluids.

TABLE 2

Biochemical determinations on trocar puncture and drainage bovine embryonic fluids, commercial BAF,[1] and on medium 199 [2]

EMBRYO CROWN-RUMP LENGTH [3]	NON-PR. N_2	PHOS.	CALCIUM	SODIUM	CHLORIDE	POTASS.	CO_2
cm	*mg %*	*mg %*	*mg %*	*m Eq./l*	*m Eq./l*	*m Eq./l*	*m Eq./l*
15.0	44	7.1	13.4	96	83	22.3	
17.0	66	4.8	19.6	62	57	35.0	
18.0	15	1.7	3.8	129	108	20.8	
21.5	175		13.9	25	7	10.8	
24.5	25	1.9	4.3	126	121	16.5	
26.0	31	5.7	5.2	121	107	20.5	
Average	59	4.2	10.0	93	80	21.0	
Stand. dev.	54	2.1	5.9	38	39	7.3	
Commercial BAF	21	1.9	5.9	118	111	14.0	13.8
Medium 199	17	2.6	4.4	146	140	2.6	7.8

[1] Obtained from Cudahy's, Omaha, Nebraska. Two different lots were tested; values were almost identical, so the average is given.

[2] With 3.0 ml of 2.8% sodium bicarbonate added per 100 ml. Values given are the averages of determinations on two different lots.

[3] These embryos would represent gestational ages of approximately 85 to 115 days, or weights of about 200 to 650 grams (Swett, Matthews, and Fohrman, '48).

Chemical determinations on the 6 trocar puncture and drainage fluids gave values generally intermediate between those found for allantoic and amniotic fluids. These results are presented in table 2, along with comparative data for commercial bovine amniotic fluid and Medium 199. Tests for protein were also done with many of the specimens, but results are not included above. Protein values were uniformly low, less than 50 mg%.

Buffering capacity. A number of specimens of bovine amniotic fluids were titrated with 1 N HCl to determine the

nature of buffers present. The initial pH of freshly collected fluids stored overnight in the refrigerator in tightly stoppered flasks varied considerably, ranging between pH 7.1 and 7.8. Similar titration curves for Medium 199, containing the usual concentration of bicarbonate, as well as greater amounts, were also determined. These data are shown in figure 1.

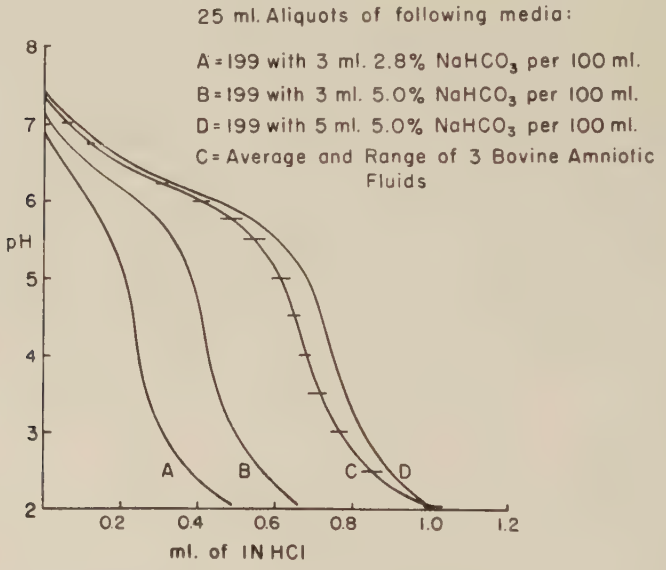

Fig. 1 Titration with HCl of bovine amniotic fluid compared with Medium 199 containing increasing amounts of bicarbonate.

DISCUSSION

Chemical values for bovine amniotic and allantoic fluids were probably not significantly altered by the degree of cell contamination encountered. However, these fluids may contain cells in numbers sufficient to warrant measures for their exclusion when bovine embryonic fluids are used as cell culture media.

It should be emphasized that the chemical determinations herein reported apply to the fluids as obtained for tissue culture purposes, and may not reflect the same environment in the intact animal. The possible biochemical changes in-

duced by the agonal state during slaughter and subsequent lapse of several hours before the uteri were sampled is not known. However, our values obtained for certain constituents in bovine embryonic fluids are quite similar to the results of Malan, Malan, and Curson ('37) for the sheep, particularly with respect to the wide variation and general magnitude of values. Some of the differences between amniotic and allantoic fluids are probably related to urine excretion into the allantois of the developing embryo. The results of determinations with blind puncture and drainage fluids suggest that material obtained in this manner consists of a mixture of both amniotic and allantoic fluids, even though the fluid is clear and apparently of amniotic origin on the basis of color. These data also indicate that considerable latitude in concentration of certain ionic constituents is possible in culture media for mammalian cells. For example, the potassium content of many bovine amniotic fluids was 4– to 8–fold higher than that of Medium 199. Yet, confirming Enders' original report ('53), in an experience involving hundreds of different samples of bovine embryonic fluids, this material has consistently produced good growth of a variety of human tissues.

The titration curve for bovine amniotic fluid is compatible with a primary buffering effect of bicarbonate. This was supported by the fact that increasing the bicarbonate in Medium 199 resulted in a titration curve very similar to that of the bovine amniotic fluid, and also by demonstration in amniotic and allantoic fluids of bicarbonate in concentrations approximating that in human serum. Thus, the relatively slow fall in pH in cultures nourished by bovine embryonic fluids, as compared to most synthetic culture media, may be partially explained by these observations. The nature of the carbohydrates present may also influence the pH changes of such fluids when used as culture medium, since it has been reported (Eagle et al., '58) that differences in utilization and consequent lactic acid production occur with various sugars in tissue culture media.

SUMMARY

Data are presented on the content of protein, non-protein nitrogen, phosphorus, calcium, sodium, potassium, chloride, and bicarbonate occurring in bovine amniotic and allantoic fluids used for tissue culture work. Wide variation in values for many of the constituents was encountered. The chemical studies suggest that fluid obtained by blind puncture of the gravid uterus is likely to be a mixture of amniotic and allantoic fluids. Study of the titration curves indicated that the principal buffer in bovine amniotic fluid was bicarbonate.

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LITERATURE CITED

Ayer, J. B., M. E. Dailey and F. Fremont-Smith 1931 Denis-Ayer method for the quantitative estimation of protein in the cerebrospinal fluid. Arch. Neur. Psychiat., *26:* 1038.

Collip, J. B. 1927 Some observations on the composition of the blood and tissues of the foetal calf. Trans. Royal Soc. Canada, *21:* 147.

Conklin, R. L., J. B. McCarthy, R. R. Thompson and L. I. Pugsley 1931 Clinical, bacteriological, and physio-chemical studies of the pregnant bovine uterus. Cornell Vet., *21:* 177.

Döderlein, A. 1890 Vergleichende Untersuchungen über Fruchtwasser und fötalen Stoffwechsel. Arch. fur. Gynekol., *37:* 141.

Eagle, H., S. Barbon, M. Levy and H. O. Schulze 1958 The utilization of carbohydrates by human cell cultures. J. Biol. Chem., *233:* 551.

Enders, J. F. 1953 Bovine amniotic fluid as tissue culture medium in cultivation of poliomyelitis and other viruses. Proc. Soc. Exp. Biol. Med., *82:* 100.

Fiske, C. H., and Y. Subbarow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375.

Gürber, A., and D. Grunbaum 1904 Über das Vorkommen von Lävulose un Fruchtwasser. Munchener med. Wochenschr., No. 9 (51 Jahrgang), 377.

Malan, A. I., A. P. Malan and H. H. Curson 1937 The influence of age on (a) amount and (b) nature and composition of the allantoic and amniotic fluids of the merino ewe. Onderstepoort J. Vet. Sci., *9:* 205.

Malherbe, H. 1954 Cultivation of human tissues in media containing bovine allantoic and amniotic fluids. Proc. Soc. Exp. Biol. Med., *86:* 124.

Paton, D. N., B. P. Watson and J. Kerr 1907 On the source of the amniotic and allantoic fluid in mammals. Trans. Royal Soc. Edinburgh, *46:* 71.

PETERS, J. P., AND D. D. VAN SLYKE 1932 Quantitative Clinical Chemistry, Vol. II, Methods. Williams and Wilkins Co., Baltimore, Chap. X, XXX.

SALK, J. E., J. S. YOUNGNER AND E. N. WARD 1954 Use of color change of phenol red as the indicator in titrating poliomyelitis virus or its antibody in a tissue-culture system. Am. J. Hyg., *60:* 214.

SWETT, W. W., C. A. MATTHEWS AND M. H. FOHRMAN 1948 Development of the fetus in the dairy cow. U. S. Dept. Agr. Tech. Bull., No. 964, 1.

TISDALL, F. F. 1923 A note on the Kramer-Tisdall method for the determination of calcium in small amounts of serum. J. Biol. Chem., *56:* 439.

VAN SLYKE, D. D., AND J. M. NEILL 1924 The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. Ibid., *61:* 523.

PENETRATION OF GLUCOSE INTO THE HUMAN RED CELL: THE RECENT ATTACK ON THE CARRIER HYPOTHESIS [1]

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TWO FIGURES

A sizable body of evidence accumulated during the last decade indicates that simple sugars pass into the human erythrocyte not by simply diffusing through the cell membrane, but by forming a transitory combination with some special surface component (reviewed by LeFevre, '54, and Wilbrandt, '54; confirmed by Widdas, '54; Bowyer and Widdas, '56, and Reinwein et al., '57). Mawe ('56) rejects the kinetic aspects of this evidence, citing new densimetric and chemical experiments as proof that the entry of glucose into properly handled red cells is adequately described by the laws of diffusion. The present note challenges this conclusion on the grounds that (1) recalculation from Mawe's hematocrit and chemical analytic data supports the carrier interpretation; and (2) new experiments incorporating Mawe's procedural recommendations still contradict his densimetric results.

The chemical experiments

Mawe rapidly separated the cells and plasma at intervals after adding glucose-enriched plasma to concentrated whole blood, and determined the hematocrit and plasma glucose levels. The one complete experiment described (fig. 7 of Mawe's paper) is kinetically far more consistent with the

[1] Research supported by the U. S. Atomic Energy Commission.

carrier interpretation than with the laws of diffusion. This is readily apparent by inspection of figure 1; here Mawe's data are displayed against the alternative patterns of theoretical curves predicted by (1) Jacobs's diffusion equation given in Mawe's report, and (2) Widdas's ('52) general carrier kinetics:

$$\frac{dS}{dt} = k' \left(\frac{C_e}{K_s + C_e} - \frac{C_i}{K_s + C_i} \right),$$

where C_e and C_i are the extra- and intracellular sugar concentrations, and K_s is the carrier-complex dissociation con-

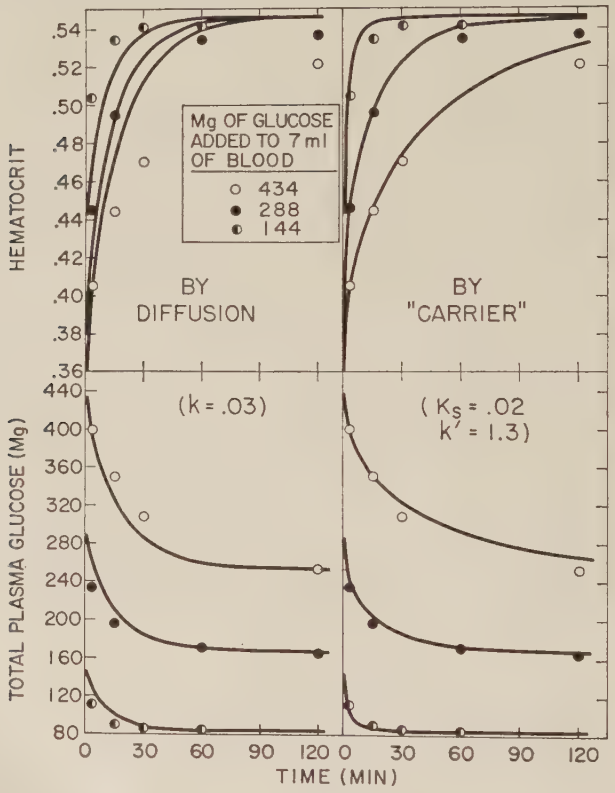

Fig. 1 Data of Mawe's figure 7 fitted to alternative theoretical predictions. Hematocrits above, glucose analyses below. Curves on left are those given by Jacobs's equation; on right, by carrier equation in text. Choice of k and k' is such as to give approximately best overall fit; (inconsistencies between hematocrit and chemical data make precise fit impossible by any interpretation).

stant, here taken as 0.02 (i.e., 0.006 M, the order of magnitude generally reported). Upon conversion to the terms used in Jacobs's equation, and integration, this yields the relation:

$$\frac{k'\mathbf{K}_s t}{1+\mathbf{Q}} = a(1-a)(\mathbf{Q}+\mathbf{K}_s)^2 \ln \frac{a-\overline{V}_o}{a-V} - (1-2a)(\mathbf{Q}+\mathbf{K}_s)(\mathbf{Q}+\mathbf{K}_s+1)(V-\overline{V}_o) - (\mathbf{Q}+\mathbf{K}_s+1)^2 \frac{(a-\overline{V}_o)^2-(a-V)^2}{2}.$$

(This differs from the diffusion equation only by introduction of the coefficients in bold-face type.)

Mawe applied Jacobs's equation to the three-minute and 15-minute readings in such experiments to calculate the *k's* given in his tables 1 and 2; he recognized the tendency of these "constants" to decrease with increasing glucose concentration and increasing time, but concluded that this was not an especially meaningful deviation. However, in the one full experiment given, this systematic variation in the "constant" is much more distinct than is shown by any of the entries in Mawe's summary tables. Recalculations from this experiment are given in table 1. The variation of k over the approximately threefold range of glucose concentrations here

TABLE 1

Relative "permeability constants" recalculated from Mawe's figure 7

Calculations are based on Jacobs's equation, with Mawe's assumptions of 0.92 as the volume fraction of water in plasma, 0.55 as the volume fraction of effective water in red cells, and initial cell glucose of zero; use of any other reasonable figures does not seriously change the relative k's.

TIME	MG OF GLUCOSE ADDED TO 7 ML OF BLOOD					
	144	288	434	144	288	434
min.	ESTIMATED ORDINATES IN MAWE'S FIGURE					
	HEMATOCRITS			TOTAL MG PLASMA GLUCOSE		
3	0.504	0.448	0.403	112	233	399
15	0.535	0.495	0.445	91	196	351
30	0.542	—	0.471	87	—	308
	CORRESPONDING "CONSTANTS" (JACOBS'S k)					
3	0.102	0.057	0.033	0.085	0.081	0.028
15	0.053	0.031	0.017	0.049	0.042	0.018
30	0.038	—	0.014	0.034	—	0.020

is in good accord with the pattern found by Wilbrandt et al. ('47) over a much wider range.

Direct comparison of the absolute figures with those graphed by Wilbrandt (as in Mawe's fig. 8) requires conversion of sugar concentrations from molarities to isosmotic units, and multiplying Jacobs's k's by 2.4 (vol. of blood water per vol. of cell water) to comply with Wilbrandt's units. When this is done, Mawe's recalculated constants are only slightly lower than Wilbrandt's. Moreover, this small difference may possibly derive from the unspecified bases taken for Wilbrandt's calculations, since the dynamics of the carrier process differ from those of diffusion and will lead to a progressive fall in the apparent diffusion k as the experiment proceeds, as illustrated in table 1.

The densimetric experiments

In Mawe's figures 3, 4, 5, and 6, the points at early intervals and at lower concentrations are notably higher than predicted. These deviations from the simple diffusion pattern in the densimetric data are in the proper direction, but in this case are not nearly large enough, to accord with the carrier kinetics expected from earlier studies. Therefore, similar experiments were undertaken to evaluate the possibility that the contrary results might arise from Mawe's technical refinements on the methods previously used by the author, in particular (1) use of defibrinated blood within 4 hours of drawing, (2) rolling of the fresh blood on an ice bath as the experiments proceeded, (3) avoidance of possibly excessive "osmotic shock" by adding blood directly to the final glucose-saline mixture (in lieu of adding hypertonic glucose to a cell suspension in saline), and (4) use of Mawe's simple phosphated NaCl medium instead of a balanced salt solution with any of a variety of organic buffers.

However, none of these technical modifications led to any deviation from the behavior found previously; the new experiments are thus at odds with Mawe's observations in appar-

ently identical situations. The example in figure 2 is nominally exactly the same experiment as in Mawe's summary figure 6; this is the densimetric record of the volume changes when cells were exposed to glucose at $\frac{1}{3}$, $\frac{2}{3}$, and 1 $\times$ isosmotic, at a constant total osmotic pressure of about twice isotonicity. The alternative theoretical patterns in figure 2 illustrate the

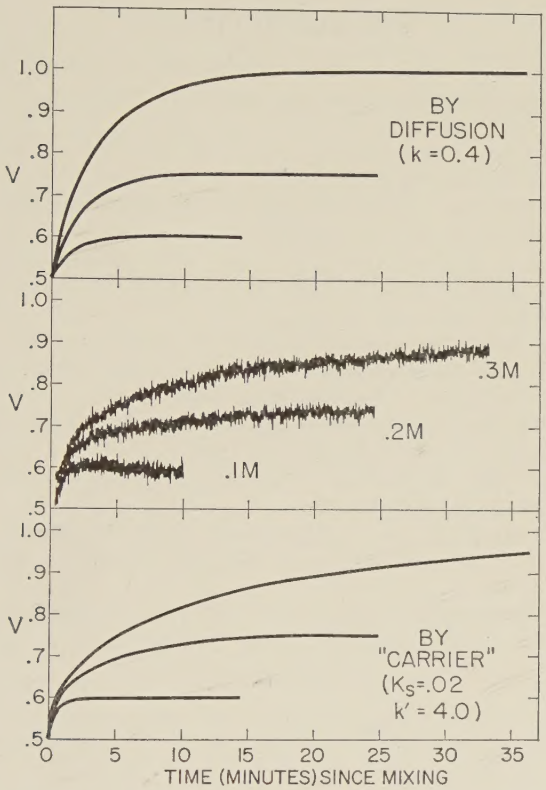

Fig. 2 Densimetric record of glucose penetration at total tonicity of 2 $\times$, at 38°C; and alternative theoretical predictions. Calculations ignore initial blood glucose and correction for displacement of water by glucose in solutions (i.e., conversion from molarities to molalities). k and k' are chosen to give fair approximation to middle experimental curve. Since V here refers to cell water volume (with volume at isotonicity as unit), k of 0.4 corresponds to Schiødt k of 0.22, about 40% higher than figure selected by Mawe, and similarly the carrier k' of 4 corresponds to 1.6 in the units of text equation. The combined constant $k'K_s = 0.08$, in good accord with the figure of 0.07 given by Wilbrandt et al. ('56).

unfitness of the diffusion interpretation, and the reasonable adequacy of the Widdas kinetics.

No clear basis is suggested for the divergent results of Mawe's densimetric experiments; the carrier kinetics have been encountered in all other systematic inquiries reported in the literature, as well as in the other phases of Mawe's own studies.

LITERATURE CITED

Bowyer, F., and W. F. Widdas 1956 The facilitated transfer of glucose and related compounds across the erythrocyte membrane. Faraday Soc. Disc., *21:* 251–258.

LeFevre, P. G. 1954 The evidence for active transport of monosaccharides across the red cell membrane. Symp. Soc. Exp. Biol., *8:* 118–135.

Mawe, R. C. 1956 The diffusion of glucose into the human red cell. J. Cell. and Comp. Physiol., *47:* 177–213.

Reinwein, D., C. F. Kalman and C. R. Park 1957 Transport of glucose and other sugars across the cell membrane of the human erythrocyte. Fed. Proc., *16:* 237.

Widdas, W. F. 1952 Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer. J. Physiol., *118:* 23–39.

———— 1954 Facilitated transfer of hexoses across the human erythrocyte membrane. J. Physiol., *125:* 163–180.

Wilbrandt, W. 1954 Secretion and transport of non-electrolytes. Symp. Soc. Exp. Biol., *8:* 136–162.

Wilbrandt, W., S. Frei and T. Rosenberg 1956 The kinetics of glucose transport through the human red cell membrane. Exp. Cell Res., *11:* 59–66.

Wilbrandt, W., E. Guensberg and H. Lauener 1947 Der Glukoseeintritt durch die Erythrocytenmembran. Helv. Physiol. Acta, *5:* C20–C22.

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